OBSERVATIONS ON THE BIOSYNTHESIS OF THE THYMINE NUCLEOTIDES AND THE THYMINE COMPONENT OF DNA.

With particular reference to the inhibitory effects of the thymidine analogue 5-iodo-2'-deoxyuridine.

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by

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C O N T E N T S.

FOREWORD.

The search for an effective cure for cancer has led to a great deal of research in many fields. With increasing knowledge of cellular metabolism it has been possible to devise drugs in an attempt to interfere with certain metabolic reactions which will bring about the death of the malignant cell. Of particular interest has been recent progress concerning the elucidation of the nature, function and synthesis of the genetic material of the cell, deoxyribonucleic acid (DNA). The biosynthetic pathways involved in the synthesis of DNA are now known in considerable detail and attempts have been made to interfere with certain of the essential reactions involved. It was the purpose of this work to investigate more fully some of the reactions involved in the biosynthesis of the thymine component of DNA and to elucidate the mechanism of action of the thymidine analogue, iododeoxyuridine, which was known to inhibit its synthesis. During the course of the investigation, iododeoxycytidine was synthesised. This compound is closely related to iododeoxyuridine and differs from it only by the presence of an amino group in lieu of an oxygen in position 6 of the pyrimidine ring and following enzymic deamination, the compound is converted into iododeoxyuridine. Investigations were performed with iododeoxycytidine in an attempt to assess the comparative effectiveness of this compound and iododeoxyuridine as inhibitors of the synthesis of the thymine component of DNA.

The antimetabolites under study were both synthesised for

the first time in the Department of Pharmacology at Yale University and leave of absence from the University of Edinburgh was granted to me for one year in order that I might carry out experimental studies in that department. During that time I was working under the close supervision of Dr. W.H. Prusoff and for the many kindnesses shown to me, by both Dr. Prusoff and the head of the department, Professor A.D. Welch, I am deeply grateful.

The author wishes to express his thanks to Dr. R.H. Girdwood on whose suggestion this thesis was initiated and whose encouragement and advice during the preparation of the manuscript have been invaluable.

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INTRODUCTION.

Deoxyribonucleic acid (DNA) exists in nature as deoxyribonucleoprotein (DNP) complexes. Both DNA and ribonucleic acid (RNA) are normal constituents of all cells both plant and animal, the DNA being confined to the nucleus, whereas RNA is found in addition in the cytoplasm. DNA is a large polymer with a molecular weight of approximately 6,000,000. Studies with the electron microscope and X-ray diffraction photographs induced Watson and Crick (1953) to postulate that the struction of the DNA molecule is a double helix consisting of two strands. There are two spirally rising chains winding around the same axis made up of phosphate groups and sugar molecules and these are linked by a series of horizontal members, like steps, consisting essentially of four bases, adenine, thymine, guanine and cytosine, arranged in pairs. By making a scale model they were able to show that the bases could fit only if they were arranged in pairs of one purine and one pyrimidine, the purine adenine and the pyrimidine thymine constituting one pair; while guanine and cytosine make up the second pair. The bases are held together by hydrogen bonding and it has been suggested that the sequence of the pairs along the chain may govern the characteristics of the cell (Crick et al, 1961).

Before a cell can undergo mitotic division, the chromosones, with their genes, must replicate and since these are composed of DNP it follows that new DNA synthesis must occur. Interference with any of the reactions essential for the synthesis of DNA should therefore inhibit the reproductive capacity of the cells. Of the

four bases which are commonly found in DNA, thymine is the only one unique to DNA, the other three, together with uracil being found also in RNA. For this reason a good deal of attention has been attracted towards the inhibition of the synthesis of the thymine component of DNA and hence of DNA itself. Although other approaches to the problem have been made, it is this one with which this thesis is mainly concerned.

NUCLEIC ACID BASES AND THEIR DERIVATIVES.

The structure of the component parts of the nucleic acid molecule is of interest, Complete hydrolysis yields pyrimidine and purine bases, a sugar component and phosphoric acid. Partial hydrolysis yields compounds known as nucleosides and nucleotides. Pyrimidine bases are all derivatives of the parent compound pyrimidine and the structure of the main ones are shown in figure 1. Cytosine and thymine are found in DNA but in RNA uracil replaces thymine. There are some minor exceptions to this rule, however, and thymine may be found in small amounts in the RNA of certain microorganisms (Littlefield and Dunn, 1958). 5-methylcytosine may also occur in small quantities in DNA from various sources (Davidson, 1960).

Purine bases are derived from the parent compound which is formed by the fusion of a pyrimidine and an imidazole ring. Adenine and guanine are the two main ones and both are found in RNA and DNA. Derivatives other than these have been found in small amounts in certain kinds of DNA and RNA.

Figure 1.

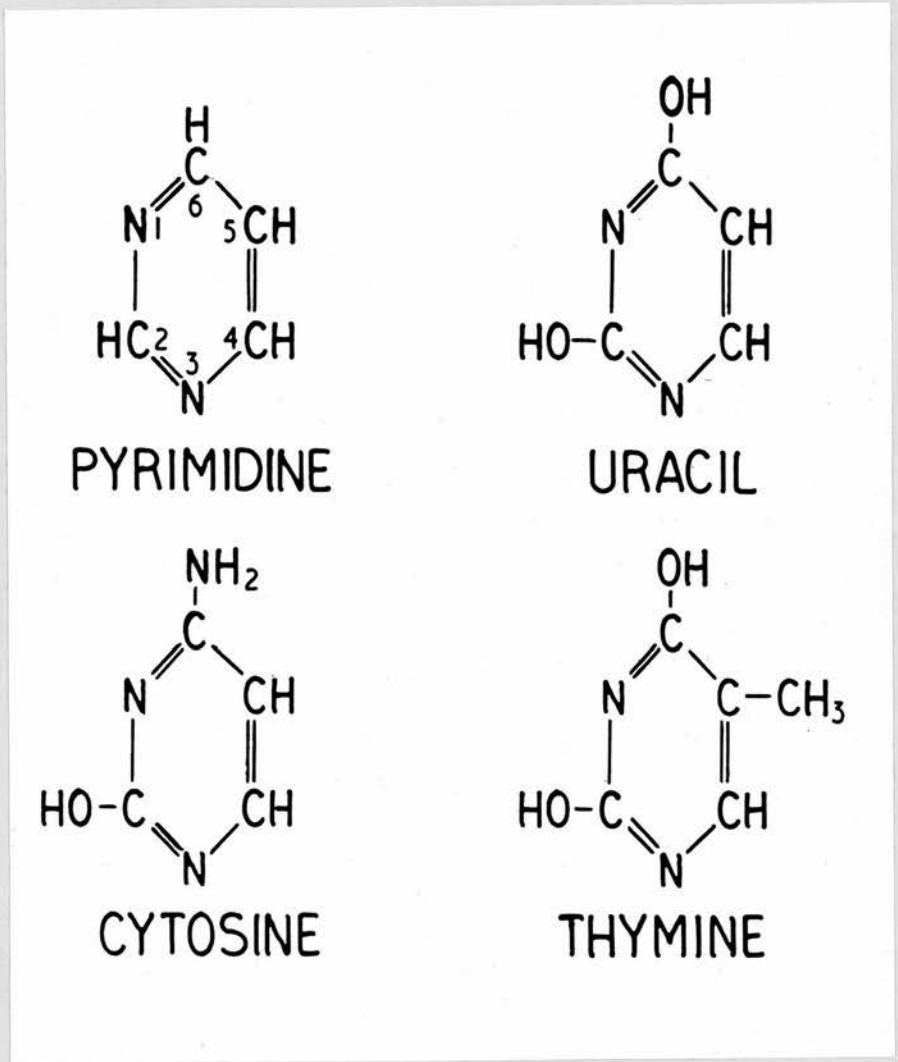

Diagram showing the structure of pyrimidine and some of the more common pyrimidine bases.

Nucleosides.

A purine or pyrimidine base may be condensed with a pentose or deoxypentose sugar to form a nucleoside. Thus thymine condenses with deoxyribose to form thymidine and uracil condenses with ribose to form uridine and with deoxyribose to form deoxyuridine.

The sugar component of RNA from every source so far tested has been shown to be D-ribose and it is now widely assumed that the pentose of RNA is always in this form.

From all mammalian and almost all other sources of DNA investigated the sugar present has been shown to be D-deoxyribose but glucose occurs glycosidically linked to hydroxymethyl cytosine in the DNA from certain strains of bacteriophage.

Nucleotides.

The nucleotides are phosphoric esters of the nucleosides. Those derived from ribose nucleosides may be called ribonucleotides and those derived from deoxyribonucleosides are deoxyribonucleotides. The ribose nucleosides have three free hydroxyl groups and therefore three possible monophosphates may be formed depending upon whether the sugar is phosphorylated at the 2', 3' or 5' positions, whereas the deoxyribonucleosides have only two free hydroxyl groups (3' and 5'). Moreover, the nucleoside 5' phosphates may be further phosphorylated at the 5' position to yield di- and tri- phosphates. Thus thymidine may be converted to thymidine-5'-monophosphate (thymidylic acid, TMP), thymidine 5'-di-phosphate (TDP) or thymidine 5'-tri-phosphate (TTP).

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CHAPTER I.

BIOSYNTHESIS OF THE THYMINE COMPONENT OF DNA.

INTRODUCTION.

This topic has been the subject of excellent reviews by both Crosbie (1960) and Reichard (1959) and reference has been made extensively to both of these accounts.

The thymine component of DNA may be synthesised de novo from small molecules within the body or from preformed pyrimidine in the form of thymidine. It is now fairly well established that none of the pyrimidines is synthesised as such but rather as the corresponding nucleotides. In the de novo synthesis the first compound to be formed with a pyrimidine structure is orotic acid (uracil-6-carboxylic acid) which is derived via carbamyl phosphate from CO_2, NH_3, ATP and aspartic acid. Uridine-5'-phosphate (uridylic acid, UMP) is synthesised following the addition of ribose-5'-phosphate to orotic acid and subsequent decarboxylation of the resultant compound (orotydylic acid). The ribose component of UMP is then reduced to deoxyribose to form deoxyuridylic acid (dUMP) and subsequent methylation at position 5 of the pyrimidine ring results in the formation of TMP.

DNA thymine may also be synthesised directly from the nucleoside thymidine by the appropriate kinases.

After the formation of TMP both pathways proceed together, the TMP being further phosphorylated to form TTP which, under the action of a polymerase enzyme is incorporated into DNA as TMP.

It is also known that cytidine and deoxycytidine can act as

precursors for the formation of DNA thymine and it is thought that these substances do do by first undergoing conversion to deoxycytidine-5'-phosphate (deoxycytidylic acid, dCMP) with subsequent deamination at position six and methylation at position five to form TMP. The exact sequence of these reactions is still not clear and will be discussed in more detail later. For the purpose of this thesis the cytosine derivatives may be regarded as entering the de novo pathway for the synthesis of DNA-thymine. It is not known whether normally synthesis from small molecules or from preformed thymidine predominates but it is known that the methylation of dUMP as judged by ^{14}C-formate incorporation into DNA-thymine is suppressed in the presence of thymidine in vitro (Prusoff et al, 1956).

The biosynthetic pathways outlined in this introductory passage will now be considered in more detail.

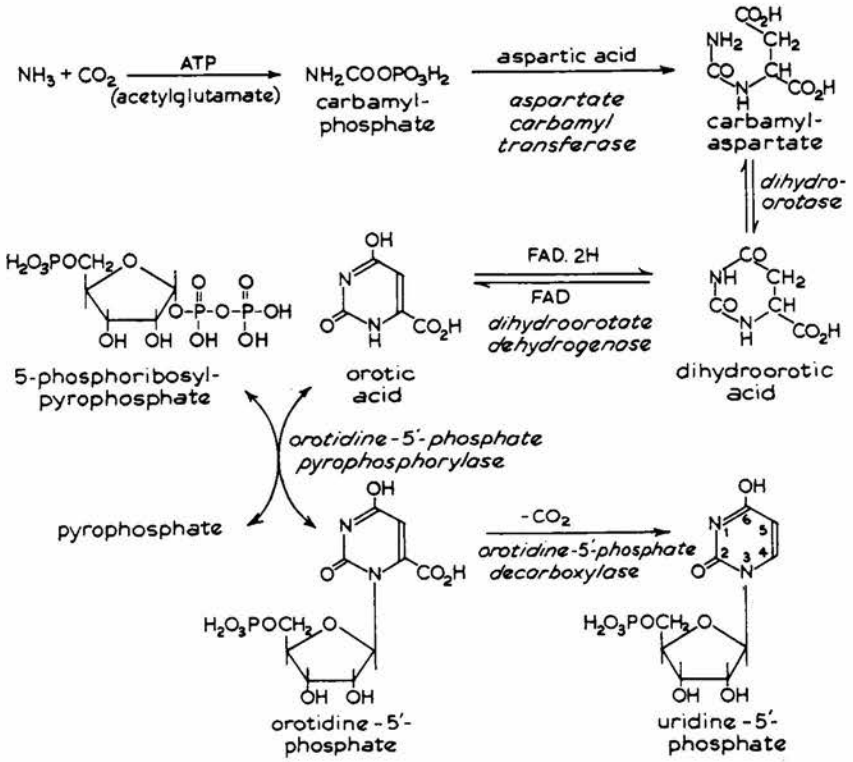
THE DE NOVO SYNTHESIS OF DNA-THYMINE.

THE BIOSYNTHESIS OF THE URIDINE NUCLEOTIDES.

a) From small molecules.

The biosynthetic pathway for the de novo synthesis of uridine-5'-phosphate is shown in figure 2 which is taken from the review by Crosbie (1960). N_1 of the pyrimidine ring has been shown to be derived from NH_3 (Lagerkvist, 1953), C_2 from CO_2 (Heinrich and Wilson, 1950) and the remainder of the ring (N_3, C_4, C_5 and C_6) is derived from aspartic acid (Lagerkvist et al, 1951; Reichard and Lagerkvist, 1953).

Each step in the synthesis will be considered briefly in turn.



The pathway of *de novo* synthesis of uridine-5'-phosphate.

Figure 2. (after Crosbie, 1960).

The first reaction involves the interaction of CO_2 and ammonia in the presence of ATP and in mammalian systems of acetyl glutamate (Hall et al, 1958) to form carbamyl phosphate (Jones et al, 1955).

The carbamyl phosphate then reacts with aspartic acid under the influence of aspartate carbamyl transferase to form carbamyl aspartate (Schulman and Badger, 1954; Smith and Stetton, 1954; Heinrich et al, 1954).

The interconversion of carbamyl aspartic and orotic acid involves the formation of dihydroorotic acid, and in cell free extracts

the enzymes dihydroorotase and dihydroorotic dehydrogenase which catalyse respectively the interconversion of carbamyl aspartic and dihydroorotic and orotic acid, (Lieberman and Kornberg, 1953; Yates and Pardee, 1956a).

Orotic acid is converted to UMP following condensation with 5-phosphoribosyl-1-pyrophosphate (Kornberg et al, 1955) by means of the enzyme orotidine-5'-phosphate pyrophosphorylase (Lieberman et al, 1955) to yield orotidine-5'-phosphate which in turn is converted to UMP irreversibly by orotidine-5'-phosphate decarboxylase (Kornberg et al, 1955; Hurlbert and Reichard, 1955).

UMP may be further phosphorylated to uridine-5'-diphosphate (UDP) and uridine-5'-triphosphate (UTP) (Potter et al, 1954) and it has been reported that cytoplasmic fractions from rat liver can bring about this phosphorylation, which is dependent upon oxidative phosphorylation reactions or the presence of ATP (Herbert et al, 1955).

The control of pyrimidine nucleotide biosynthesis in E.coli by feed back inhibition has been demonstrated (Yates and Pardee, 1956b). Cytidine and cytidine-5'-phosphate (CMP) are competitive inhibitors with aspartic acid and carbamyl phosphate for carbamyl aspartic formation. Uracil, uridine and UMP do not inhibit appreciably.

b) From preformed pyrimidines.

The ability of tissue to incorporate free pyrimidines into polynucleotides seems to vary with the tissue. The utilisation of uracil and cytosine is negligible in rat liver unless supplied in high extracellular concentration (Bendich et al, 1949) but uracil is readily incorporated into the RNA of hepatomas induced by

2-acetylaminofluorine (Rutman et al, 1954) and in the Flexner Jobling carcinoma as well as in the normal rat intestinal mucosa (Heidelberger et al, 1957a). Pyrimidine nucleosides and nucleotides do function as precursors of tissue polynucleotide pyrimidines (Roll et al, 1949; Hammarsten et al, 1949; Prusoff, 1958a; Hecht and Potter, 1956) but the evidence available suggests that sephosphorylation of the nucleotides occurs prior to entry into the cell. Utilisation of the resulting nucleoside then takes place (Liebman and Heidelberger, 1955).

THE BIOSYNTHESIS OF DEOXYRIBONUCLEOTIDES.

The de novo synthesis of the DNA pyrimidine nucleotides involves the transformation of a pyrimidine ribonucleotide to the corresponding deoxyribonucleotide. Thus ^{15}N-labelled cytidine and uridine are utilised for the synthesis of both DNA and RNA pyrimidines in the rat (Hammarsten et al, 1950). Moreover, following the injection into rats of ^{14}C-cytidine labelled in both the pyrimidine and ribose moieties, it has been shown that the ratio between the specific activities of cytosine and ribose in the RNA was the same as that between the specific activities of the pyrimidine and deoxyribose in the DNA (Rose and Schweigert, 1953). Similar results have subsequently been shown with CMP, UMP (Roll et al, 1956) and uridine (Reichard 1957).

Friedkin and Kornberg, 1957, have reported that in E.coli extracts no kinase activity with respect to deoxyuridine monophosphate (dUMP) is demonstrable. Recently, however, evidence has been

obtained in favour of an enzyme which catalyses the formation of deoxyuridine diphosphate (dUTP) from uridine diphosphate (UDP) (Bertani et al, 1961). Further studies suggest that dUDP may be phosphorylated to dUTP but that another enzyme which catalyses the formation of dUMP from dUTP is also present. The absence of uracil from DNA may thus not only be due to the absence of an enzyme which phosphorylates dUMP to dUTP but also to the presence of an enzyme which specifically cleaves dUTP. Figure 3 shows a possible pathway for the synthesis of DNA-thymine as suggested by Bertani et al (1961).

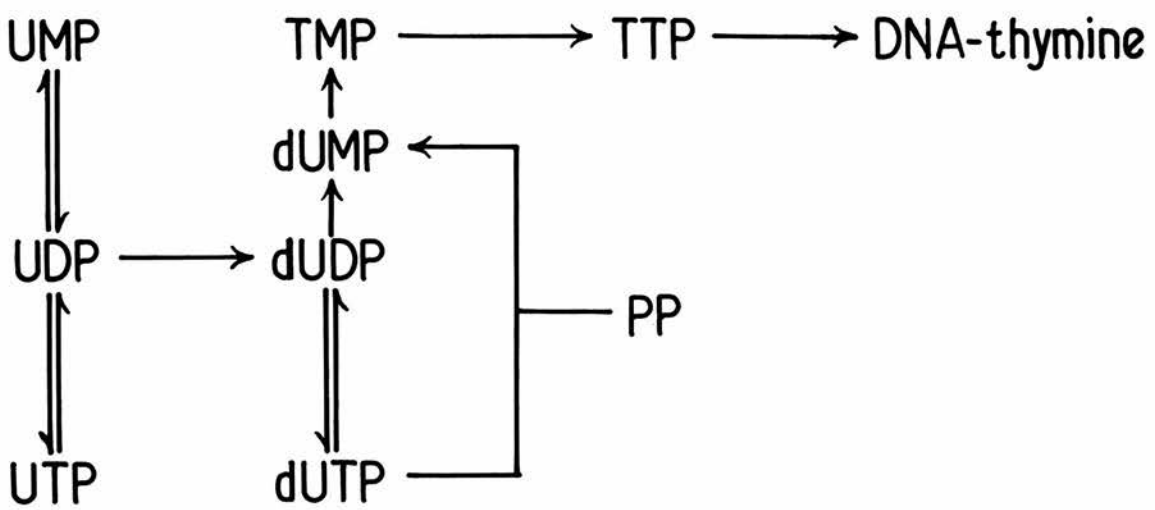
In the case of the deoxycytidine nucleotides the available evidence suggests that they are formed in the same way as the deoxyuridine nucleotides i.e. by conversion of the ribosyl to the deoxyribosyl compound at the diphosphate level (Bertani et al, 1961).

BIOSYNTHESIS OF THE METHYL GROUP OF THE THYMINE NUCLEOTIDES.

During the synthesis of thymine there occurs not only the transformation of a ribotide to a deoxyribotide but also the attachment of a methyl group to the pyrimidine ring i.e. the conversion of dUMP to TMP.

Experiments with ^{14}C-labelled compounds have shown that in mammalian cells the methyl group of thymine could originate from the beta carbon of serine (Elwyn and Sprinson, 1954), formate (Totter et al, 1951; Totter and Best, 1955), the alpha carbon of glycine (Elwyn

Figure 3.



and Sprinson, 1950), and at least in some cases, the methyl group of methionine (Herrman et al, 1955). It thus appears that the methyl group of thymine can be derived from substances which can donate a "one-carbon" unit. Crosbie (1958) has shown, however, that ^{14}C-formate is not utilised for the synthesis of the methyl group of thymine in E.coli and that in these organisms methionine is not a methyl donor.

Folic acid derivatives have been known for some time to function as co-factors of the "one-carbon" units. This was first suggested by work with microorganisms (Snell and Mitchell, 1941; Stokes, 1944; Prusoff et al, 1948) and it was demonstrated that the folic acid antagonist, aminopterin, greatly inhibited the utilisation of ^{14}C-formate for the synthesis of DNA-thymine (Totter and Best, 1955; Goldthwait and Bendich, 1952). Folic acid is first reduced to tetrahydrofolic acid (FH_4) (Handschumacher and Welch, 1960) and the N^5, N^{10} methylene derivative of this ($N^{5-10}FH_4$) behaves as an "active" one-carbon unit at the oxydation level of formaldehyde (CH_2O) (Handschumacher and Welch, 1960; Huennekens et al, 1958; Rabinowitz 1960). In order that this folic acid derivative be provided to cells the unstable FH_4 must be formed enzymatically from either folic acid or dihydrofolic acid (FH_2) or by the turnover of coenzyme molecules. FH_2 as well as being an intermediate product in the formation of FH_4 which is enzymatically active, is also the end product formed from $N^{5-10}FH_4$ when deoxyuridylic acid is converted to thymidylic acid (Friedkin 1959). The conversion of folic acid to FH_2 and FH_4 is dependent upon the enzyme folic acid reductase (Huennekens et al, 1958; Osborn et al, 1958; Peters and Greenberg, 1959; Zakrzewski and Nichol, 1960; Holland 1961) and it is this enzyme upon which the folic acid antagonists, aminopterin and amethopterin, act (Handschumacher and Welch, 1960; Holland 1961).

Either a uracil or a cytosine derivative could theoretically act as the acceptor of the "one-carbon" unit. In the first instance

methylation would give rise directly to a thymine derivative but in the case of cytosine, deamination of the formed 5-methylcytosine derivative would have to take place and in this connection it is of interest that a deaminase for 5-methyl deoxycytidine has been described (Cohen and Barner, 1957). Another point of importance is whether the methylation occurs before or after the conversion of the ribonucleotide to the deoxyribonucleotide.

In an attempt to answer these questions Reichard,(1955) designed experiments in which a comparison of the utilisation of 5-methyluridine, deoxyuridine, uridine, thymine and thymidine for polynucleotide pyrimidine synthesis in regenerating liver and intestinal mucosa was made. They revealed that deoxyuridine like thymidine is utilised almost exclusively for DNA-thymine formation. No incorporation of deoxyuridine into DNA-cytosine was noted. Uridine was utilised extensively for the synthesis of polynucleotide uracil, cytosine and thymine whereas 5-methyluridine, like thymine, showed a small but significant incorporation into DNA-thymine of regenerating liver (but not of intestinal mucosa). The evidence suggests that deoxyuridine is the primary "1-C" acceptor molecule in thymine ring biosynthesis. Friedkin and Roberts, (1956)have described a similar aminopterin inhibited incorporation of deoxyuridine into the DNA-thymine of chick embryo and bone marrow cells. Prusoff et al (1958 and 1956) have shown that deoxyuridine, deoxycytidine, uridine and cytidine increase the incorporation of ^{14}C-formate into the DNA-thymine of rabbit bone marrow and Ehrlich ascites carcinoma cells. The cytosine nucleosides are markedly more efficient than the uracil derivatives. This evidence, together with the observation

of methyldeoxycytidine deaminase activity in E.coli extracts has been interpreted as indicating the possible role of deoxycytidine (or a nucleotide derivative) as "1-C" acceptor molecule in thymine ring formation.

Studies at the enzyme level on cell free extracts have also been used to elucidate the methylation reaction. Using Dowex-1 treated extracts of E.coli it was shown that dUMP labelled with ^{32}P or ^{14}C was transformed to TMP in the presence of serine, FH_4, ATP and Mg^{++} (Friedkin and Kornberg, 1957). The same reaction was also found in mammalian tissues (Blakley, 1957; Phear and Greenberg, 1957; Humphreys and Greenberg, 1958).

The role of vitamin B_{12} in nucleic acid synthesis is still obscure. Evidence has been presented for its participation in deoxyribonucleoside synthesis (Downing and Schweigert, 1956). Others have proposed that it is concerned in "1-C" metabolism (Dinning et al, 1958; Bolinder and Reichard, 1959) being involved in the methylation reaction rather than in deoxypentose formation.

The immediate "1-C" donor appears to be N^5N^{10} methylene-FH_4 (Blakley, 1958; Kisluik, 1957). The mechanism of the methylation reaction is still uncertain. Two possibilities will be briefly mentioned. The first proposes that N^{5-10} FH_4 and dUMP, are linked, each through their respective position 5, by the "active methylene" group derived from formate (Greenberg and Humphreys, 1958; Friedkin, 1959).

Cleavage of the complex to form TMP is thought to involve the transfer of one atom of hydrogen to yield the 5-methyl group, with the separation of FH_2 (Blakley, 1957). FH_4 is then regenerated from FH_2 by the action of dihydrofolic acid reductase. The second possibility involves the hydrolysis of the complex of dUMP and N^{5-10} FH_4 to yield a 5-hydroxymethyl pyrimidine derivative which is then reduced to the 4, 5 di-hydro derivative, dehydrated and rearranged to give TMP (Hamill et al, 1956; Cohen et al, 1956).

THE BIOSYNTHESIS OF TTP FROM TMP.

The phosphorylation of TMP to the corresponding triphosphate is controlled by kinases which have been intensively studied is soluble extracts of E.coli, Ehrlich ascites tumour cells and regenerating rat liver. The enzymes responsible for the phosphorylation of thymidine and TMP to TTP are of special interest, for although their activity is very low in extracts of normal liver, it is pronounced in extracts of liver regenerating after partial hepatectomy (Bollum and Potter, 1959; Weissman et al, 1960b; Mantsovanos and Canellakis, 1959b). In this respect the thymidine nucleotide kinase system differs from the kinases involved in the phosphorylation of the monophosphates of adenine, guanine and cytidine which are of comparable activity in normal and regenerating liver. During the process of liver regeneration the kinases responsible for the phosphorylation of thymidine, TMP and TDP appear in that order, reach maximum activity at the time of maximum DNA synthesis and then decline. Similar findings were also found in cultures of the L strain of

fibroblast innoculated into fresh medium (Weissman et al, 1960).

Kornberg and his colleagues (Kornberg 1959 and 1960; Lehman, 1959) showed that cell free extracts made from cultures of E.coli contained an enzyme polymerase which would bring about the synthesis of DNA in the presence of the four predominant bases adenine, guanine, cytosine and thymine together with Mg^{++} and DNA primer. The enzyme catalysing the reaction has been purified and experiments designed to demonstrate the DNA synthesis from various isotopically labelled substrates. From such experiments it can be shown that from 90-95 per cent of new DNA has its origin in the deoxyribonucleoside triphosphate substrates. The nucleotides incorporated into DNA are formed by the 3' 5'-phosphodiester linkage and inorganic pyrophosphate is liberated. A similar polymerase system has been described in cell free extracts from Ehrlich ascites carcinoma cells (Smellie et al, 1959) regenerating rat liver (Bollum and Potter, 1959) and calf thymus (Bollum, 1960). The polymerase enzyme takes directions from the primer whose pattern it faithfully reproduces.

Since the product of polymerase action is a conventional double helix conforming to the Watson-Crick model it is not surprising to find that specific replacement of certain bases by analogues can be made provided that the correct hydrogen bonding relationships are maintained in the "fraudulent" DNA so formed (Bessman et al, 1958). Thus 5-bromuracil after conversion to the nucleoside triphosphate can replace thymine. Such analogues will be discussed more fully in the next chapter.

INTERRELATIONSHIP OF URACIL AND CYTOSINE DERIVATIVES.

A connection between cytosine and uracil derivatives is achieved through amination and deamination reactions. The conversion of UTP to CTP by an E.coli extract in the presence of NH_3 and ATP has been reported (Lieberman, 1955 and 1956) but uracil, uridine and uridylic acid were not similarly utilised. In mammalian cells however, there is evidence to suggest that the amino group of the cytosine derivative is derived from glutamine rather than from ammonia (Salzman et al, 1958).

The uracil and cytosine ring systems are also interrelated by the cytosine and cytidine deaminase described in a variety of mammalian and microbial cells but these systems are thought to be catabolic with respect to the cytosine derivatives rather than anabolic with respect to uracil and its derivatives (Simcock et al, 1957).

A deoxycytidylic acid deaminase is now known to exist in several tissues. Enzymatic deamination of dCMP to dUMP in sea urchin eggs was originally demonstrated by Scarano and Maggio (1958 and 1959) and later work with the livers of rats, chickens and rabbits in various stages of their embryonic development suggested that the deaminating activity is proportional to the growth rate (Scarano and Talarico, 1959; Maley, 1959). For example, the enzymes are essentially absent in normal rat liver but measurable in regenerating liver (Maley, 1960). These data suggest that the enzyme may have a part to play in the synthesis of new DNA. In adult tissues dCMP deaminase is less readily demonstrated particularly in the rat and

rabbit but the enzyme is present in appreciable quantities in the mucosa of the alimentary tract of the human especially in the stomach and colon (Guerritore et al, 1960).

As with the uracil derivative CMP is phosphorylated by a cytoplasmic fraction during oxidative phosphorylation to cytidine-5'-di and triphosphates (CDP and CTP respectively) (Herbert et al, 1955). CMP is formed from cytidine and inorganic phosphate in a variety of cell systems (Brawerman and Chargaff, 1954 and 1955; Tunis and Chargaff, 1956) and as no de novo pathway of cytidine (or uridine) synthesis is known to exist other than via the corresponding 5'-nucleotide, the nucleotide phosphotransferase enzyme involved may be merely that of salvage mechanism. dCMP can be phosphorylated by ATP in the presence of the cytidine monophosphate kinase described by Maley and the product further phosphorylated to dCTP by a nucleoside diphospho kinase (Maley, 1958; Lehman et al, 1959; Canellakis and Mantsovanos, 1958).

BIOSYNTHESIS OF THE THYMINE NUCLEOTIDES AND DNA-THYMINE FROM PREFORMED THYMINE DERIVATIVES.

Free thymine, like uracil and cytosine undergoes little or no utilisation (Reichard, 1955; Holmes et al, 1954; Plentl and Schoenheimer, 1944; Rutman, 1954; Bendich et al, 1949) for mammalian DNA synthesis. Thymidine, on the other hand, like other pyrimidine deoxyribonucleosides, is extensively utilised for the biosynthesis of DNA-thymine in both avian (Friedkin and Wood, 1956b; Friedkin et al, 1956c; and mammalian tissues (Maley, 1958; Greenberg and Humphreys, 1958; Friedkin & Wood, 1956b; Friedkin et al 1956c).

The pathway of thymidine utilisation has been elucidated by the observation of kinase activities in cell free preparations of mammalian (Canellakis and Mantsovanos, 1958; Bollum, 1958; Bollum and Potter, 1958; Weissman et al, 1960) and bacterial origin (Lehman et al, 1958). Thymidine is phosphorylated by thymidine kinase to TMP which is then further phosphorylated and incorporated into DNA as described above

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CHAPTER II.

AGENTS WHICH INFLUENCE THE BIOSYNTHESIS OF THE THYMINE COMPONENT OF DNA.

INTRODUCTION:

The important phenomenon of "thymineless death" of bacteria (Cohen and Barner, 1956) has, of recent years, stimulated interest in antimetabolites which might bring about such a state. Many of the compounds which have been prepared bear a close resemblance to normal metabolites except for certain minor structural alterations. For example, uracil may be modified by replacement of the hydrogen atom, in position five of the pyrimidine ring by a fluorine atom (5-fluorouracil) or the hydroxyl group in position 4 of the pteridine ring of pteroylglutamic acid may be replaced by an amino group (aminopterin). Alternatively, the ring structure itself may be altered as in azauracil and azathymine where the carbon atom in position 6 of the pyrimidine ring is replaced by a nitrogen atom. Such compounds are sufficiently similar to the normal metabolites as to participate in or inhibit certain specific metabolic reactions.

Although much can be learned from *in vitro* studies with these drugs about their site of action, *in vivo* studies are of very great importance when considering their effectiveness as chemotherapeutic agents. Some compounds, for example, act as antimetabolites only after they have undergone alteration. In the case of the halogenated pyrimidines, conversion of the analogue to the mononucleotide, or higher metabolic forms probably takes place and while this particular

reaction occurs intracellularly and can be demonstrated in *in vitro* experiments, certain other reactions such as deamination of cytosine derivatives may have to be carried out in specialised organs such as the liver and require *in vivo* studies to evaluate fully the therapeutic efficacy of the drug. Moreover, some compounds, although active, may not be transported to certain areas of the body (e.g. the brain) and their value as chemotherapeutic agents is therefore limited. Other compounds may be rapidly catabolised and excreted in the urine having been present in the body in adequate concentration for insufficient length of time to produce any significant metabolic effect. Finally, different tissues may utilise the analogue to varying degrees giving rise to a selectivity of action which *in vitro* studies would not reveal.

It should be stated, however, that while experiments with whole animals will give considerable information it is to be noted that not all species respond in the same way to any particular compound. In some instances man may respond in a way that is unique so that not until it has been administered to man himself can the drug be fully evaluated. For example, azauridine is very toxic to the bone marrow of the dog but it is thought to have no effect on the haemopoietic tissues in man.

Compounds which inhibit the synthesis of DNA-thymine fall into two main groups. Those which interfere with the *formation* of TMP and those which interfere with its *utilisation* i.e. its further phosphorylation and incorporation into DNA. Brief mention will be made of the various classes of compounds which interfere with the

synthesis of DNA-thymine but only the halogenated pyrimidines will be discussed in detail since it is with these drugs that much of the experimental work to be described later was undertaken.

COMPOUNDS WHICH INHIBIT THE FORMATION OF TMP.

FOLIC ACID ANTAGONISTS:

Aminopterin and Amethopterin.

Many derivatives of folic acid have been prepared (Seeger et al, 1947) but of these Aminopterin and Amethopterin are by far the most important. Aminopterin is derived from folic acid by the substitution of an amino group for a hydroxyl group in the 4 position and amethopterin is the N^{10}-methyl derivative of aminopterin. These compounds serve as antimetabolites by virtue of their action upon the enzyme folic acid reductase (Peters and Greenberg, 1959; Zakrzewski and Nichol, 1960; Osborn et al, 1958; Huennekens et al, 1958; Nichol and Welch, 1950) which is involved in one carbon transfer reactions not only for the synthesis of TMP but also for the biosynthesis of the first purine-containing compound, inosinic acid, which subsequently is converted into adenine and guanine containing components of nucleic acids and many coenzymes. The affinity of folic acid reductase for the 4-amino derivatives of folic acid is so great as compared with its affinity for folic acid itself or dihydrofolic acid that the effects of the drugs are long lasting and impossible to overcome by the administration of folic acid. However, early signs of toxicity may be prevented by giving folinic acid which is a relatively stable derivative of FH_4 and which supplies the product of the blocked reaction (FH_4) but does not actually release the enzyme from its inhibition

(Broquist et al, 1950; Burchenal and Babcock, 1951; Sauberlich and Baumann, 1948; Schoenbach et al, 1950).

These compounds are very effective in inducing excellent remissions in many cases of childhood leukaemia (Farber et al, 1948) and certain other types of malignancy, particularly chorion epithelioma (Hertz et al, 1958). Recent evidence suggests that their effectiveness may be related to the relatively short supply of folic acid reductase in the susceptible cells (Bertino et al, 1960; and 1961a)and that the development of resistance to the drug is related to selective reproduction of cells rich in this enzyme (Bertino et al, 1961b;Fischer 1959 and 1961)

Diaminopyrimidines and Diaminodihydrotriazines.

Several compounds with the structure of a diaminopyrimidine or a diaminodihydrotriazine have been prepared. These compounds are thought to act by interfering with the formation of a coenzyme closely related to FH_4 and therefore with the synthesis of nucleic acids (Swaffield et al, 1959). Direct studies of the effects of these agents on folic acid reductase have, however, not been made. Two antimetabolite drugs, chlorguanide and pyrimethamine are of interest in this group of compounds. The latter is a diaminopyrimidine and the former is thought to be converted to a diaminodihydrotriazine within the body. The evidence suggests that both compounds act by interfering with the formation of FH_4 or closely related compounds by certain plasmodia (Hitchings, 1952; Modest et al, 1952; Foley, 1953; Swaffield et al, 1959).

URETHANE.

Ethyl carbamate (urethane) has the capacity to affect cellular reproduction and nucleic acid metabolism but its mode of action is uncertain. It acts as an inhibitor of various transplantable neoplasms (Elion et al, 1958a;Haddow and Sexton, 1946) and is used to a limited extent in the treatment of multiple myeloma (Paterson et al, 1946; Berman and Axelrod, 1948; Loge and Rundles, 1949). Urethane has also been shown to induce neoplasms in certain strains of mice and rats and the neogenic action was reduced by the presence of thymine but less so by thymidine (Rogers 1951, 1955, 1957 (a) and 1957 (b)). Since thymine is poorly utilised for DNA-thymine synthesis and is rapidly and extensively degraded (Reichard, 1955; Prusoff et al, 1954; Fink et al, 1956) the possibility that it may be used for the formation of substances other than DNA-thymine or may exert indirect effects must be borne in mind. It has been suggested that urethane influences the formation of the pyrimidine nucleus from carbamyl phosphate and aspartic acid, and the methylation and the amination of the uracil moiety but much more work is required to explain satisfactorily the effects of this compound.

PYRIMIDINE ANALOGUES.

5-Amino-, 5-mercapto-pyrimidines.

Aminouracil is thought to exert its antimicrobial activity by acting both as an antifolic and as an antagonist to thymine (Hitchings et al, 1945 and 1950). Aminodeoxyuridine inhibits the growth of E.coli 4-12 and the inhibition is overcome best by thymidine (Beltz and Visser, 1957) suggesting that the drug inhibited

the formation of thymine nucleotides.

5-mercaptouracil effectively inhibits the growth of L.leichmannii and its action is reversed by thymine, or more effectively by thymidine (Bardos et al, 1955) in a competitive manner. When combined with fluorouracil this compound was also effective in producing significant inhibition of the growth of the animal tumours sarcoma-180 and adeno carcinoma-755 which has been interpreted as being due to multiple blockage of de novo synthesis of thymine derivatives and utilisation of preformed thymine (Bardos et al, 1959)

Azapyrimidines.

The azapyrimidines are formed by replacement of one of the ring carbons and its associated hydrogen with a nitrogen atom. Azauracil and its derivatives and azathymine are of particular interest with regard to DNA-thymine synthesis.

Azauracil and its metabolic derivative 6-azauridine have both been found to possess antimicrobial activity (Handschumacher and Welch, 1956), but azauridine is a more effective inhibitor of tumour growth (Jaffe et al, 1957; Sorm and Keilova, 1958) presumably due to the fact that mammalian cells convert azauracil to the corresponding ribosides relatively poorly (Jaffe et al, 1957). Azauridine is further converted to the nucleotide derivative (Habermann and Sorm, 1958; Pasternak and Handschumacher, 1958; Handschumacher and Pasternak, 1958; Pasternak and Handschumacher, 1959) which inhibits the enzyme orotic acid decarboxylase. As a result, orotic acid is found in the urine (Habermann and Sorm, 1958) and the de novo synthesis of RNA and DNA pyrimidines (including thymine) is

inhibited (Pasternak and Handschumacher, 1959).

Azathymine is an effective inhibitor of the growth of many organisms and the inhibition can be overcome in a competitive manner by thymine or thymidine (Prusoff et al, 1954; Elion et al, 1954). The analogue exerts its effect on organisms utilising exogenous thymine or thymidine and on those synthesizing it de novo. Azathymidine was an even more potent inhibitor than the free pyrimidine (Prusoff and Welch, 1956). Azathymidine but not azathymine will inhibit the incorporation of formate into the DNA thymine of rabbit bone marrow cells and Ehrlich ascites cells in vitro (Welch et al, 1955; Prusoff et al, 1956; Prusoff 1959) whereas incorporation of thymidine was relatively insensitive to azathymidine. These results are not strictly comparable, however, since in the former case the azathymidine was competing with what are probably only very small amounts of thymine nucleotides.

Uracil Methylsulphone.

This compound is a potent inhibitor of certain microorganisms (Holmes and Welch, 1956) and a number of tumours (Jaffe and Cooper, 1958). In Ehrlich ascites cells the uptake of formate and orotic acid into DNA thymine is inhibited though the analogue has no effect on the utilisation of preformed thymidine (Prusoff, 1958).

Fluorinated Pyrimidines.

Several compounds have now been synthesised in which the hydrogen atom at position 5 of the pyrimidine ring has been replaced by a halogen atom. In particular, the halogenated uracil compounds and thymine have similar physical and chemical properties so that a

considerable amount of work has been undertaken to elucidate the mechanism whereby these compounds influence the synthesis of DNA-thymine. Fluorine has a van der Waal's radius of 1.35 A as compared with that of hydrogen of 1.20 A, whereas that of iodine is 2.15 A. bromine 1.95 A and chlorine 1.85. It can thus be appreciated why fluorine which bears close similarities in physical and some chemical properties to hydrogen substitutes for the latter in certain biologically active compounds. Iodine and bromine, on the other hand, substitute at position 5 of the pyrimidine ring for a methyl group which has a van der Waal's radius of 2.0 A. In consequence fluorinated uracils serve as uracil analogues whereas iodinated or brominated uracils serve as thymine analogues. The chloro derivative apparently behaves as both a uracil and a thymine analogue since it is incorporated into both RNA and DNA of micro-organisms. 5-fluoro uracil was the first of the fluorinated pyrimidines to be synthesised (Duschinsky et al, 1957a)but fluoro deoxyuridine, fluoro-orotic acid (Chaudhuri et al, 1958), fluoro-cytosine (Heidelberger et al, 1957) fluoro-cytidine (Duschinsky et al, 1957) and fluoro-deoxycytidine (Duschinsky et al, 1957b Cohen et al, 1958) have all been synthesised and tested subsequently.

(a) 5-Fluorouracil (5-FU).

This compound inhibits the synthesis of the uracil and cytosine components of RNA and, following conversion to 5-fluoro-deoxyuridylic acid, that of DNA thymine as well.

It has inhibitory activity for a wide spectrum of micro-organisms (Heidelberger et al, 1957; Scheiner et al, 1957) which can be reversed by thymidine in a non-competitive manner and to a

lesser degree by uracil or cytosine. Inhibition of a variety of mouse tumours has also been observed (Heidelberger et al, 1957b and 1958a). In Ehrlich ascites tumour cells *in vivo* formate incorporation into DNA-thymine was inhibited and orotic acid incorporation into DNA-thymine and RNA-uracil was also inhibited (Heidelberger et al, 1957; Lanneberg et al, 1958). At the same time thymidine incorporation into DNA was increased suggesting that the mode of action of fluorouracil is, following its conversion to 5-fluorodeoxyuridylic acid, the inhibition of the conversion of uracil derivatives to thymine nucleotides with consequent utilisation of exogenous thymidine. Inhibition of the thymidylate synthetase reaction has also been demonstrated in a cell free system (Heidelberger and Hartmann, 1961; Farkas et al, 1956). That fluorouracil also effects the synthesis of RNA-pyrimidines was evident from the fact that in the same dosage it produced up to 68 per cent inhibition of the incorporation of uracil or orotic acid into the uracil or cytosine of RNA (Heidelberger et al, 1957b; Lanneberg et al, 1958).

In vitro studies have shown that in general fluorouracil undergoes all the anabolic reactions of uracil (Harbers et al, 1959; Chaudhuri et al, 1958; Bosch et al, 1958), with the exception of methylation at position 5 of the derivative corresponding to dUMP, this site having been blocked by the fluorine atom. Incorporation of fluorouracil into the RNA of many mouse and human tissues also occurs (Chaudhuri et al, 1958). Whether it replaces any specific RNA base cannot definitely be stated but no significant incorporation

of the analogue in the form of fluorocytosine has so far been noted in the RNA or DNA of mammalian or bacterial cells. Since fluorouracil inhibits the incorporation of both orotic acid and uracil into RNA-pyrimidines it has been postulated that it inhibits an enzymic site of action at the ribonucleotide level beyond UMP but proof of this is still awaited (Welnick et al, 1958).

As a result of this disordered metabolism interesting changes may be observed in growing cells by the use of cytochemical techniques (Lindner, 1958 and 1959). In Ehrlich ascites cells treated with fluorouracil there was a reduction in the mitotic index with enlargement of the cells which contained an increase in the amount of protein and RNA within the cells. The DNA content was reduced by 50 per cent. Feulgen staining of interphase cells demonstrated coarse clumps and strands of DNA around the nucleus indicating early death.

Of considerable interest with regard to this compound are the reports indicating marked differences in mammalian tissues in the capacity to utilise preformed uracil (Rutman et al, 1954; Heidelberger et al, 1957a; Lagerkvist and Reichard, 1957; Reichard and Skold, 1958). For example, the analogue was found to inhibit the incorporation of ^{14}C-formate into the thymine of DNA in Ehrlich ascites cells preferentially as compared with spleen, liver or intestines. Moreover, preferential concentration of the fluorouracil derivatives in a number of experimental tumours and in human neoplastic tissues following its administration in vivo has been noted (Chaudhuri et al, 1958).

(b) 5-Fluorouridine and 5-Fluorodeoxyuridine.

These compounds have many biological properties in common with

fluorouracil (Harbers et al, 1959; Bosch et al, 1958). Fluorodeoxyuridine, however, has an even greater affinity for tumour (sarcoma-180) than fluorouracil while fluorouridine has less (Harbers et al, 1959). Fluorodeoxyuridine has a greater inhibitory effect on tumours in vivo (Heidelberger et al, 1958) and it causes a "thymineless" death of E.coli but its length of action is limited owing to its degradation to fluorouracil by deoxyribonucleosides (Cohen et al, 1958).

Since the mechanism of action of fluorouracil and its deoxyriboside involves an inhibition of the thymidylic acid synthetase system it would be logical to expect that thymidine might antagonise their action as a result of the formation of TMP by the action of thymidine kinase. This is the case with fluorodeoxyuridine if thymidine is administered intravenously simultaneously with the analogue (Miller et al, 1961) but in certain circumstances, thymidine actually increases the toxicity of the drug (Burchenal et al, 1959aand 1960). This may be attributed to the rapid catabolism of both compounds. Thus thymine is released from thymidine and fluorouracil from fluorodeoxyuridine. Thymine then competes with fluorouracil for further catabolic enzymes and thus permits increased utilisation of the analogue with a consequent increase in toxicity.

(c) 5-Fluoroorotic Acid.

This compound behaves in a manner similar to fluorouracil particularly with reference to synthesis of DNA pyrimidines (Heidelberger et al, 1957) but it is not utilised preferentially by tumours (Chaudhuri et al, 1958). In addition, fluoroorotic acid

inhibits the synthesis of orotidylic acid from orotic acid and 1-pyrophosphorylribose-5-phosphate (Stone and Potter, 1957).

(d) 5-Fluorocytosine, 5-Fluorocytidine and 5-Fluorodeoxycytidine.

Fluorocytosine has little biological activity but the corresponding riboside and deoxyriboside do reduce the rate of growth of experimental tumours (Burchenal et al, 1958 and 1959b; Eidinoff et al, 1959a). In vitro studies with Ehrlich ascites cells indicate that fluorocytidine appears to act mainly by inhibition of the incorporation of uracil and cytidine into DNA thymine (Harters et al, 1959), which suggests that the compound acts after its deamination to form the corresponding uracil derivatives, the deamination occurring either before or following its conversion to the deoxyribotide.

COMPOUNDS WHICH INHIBIT THE UTILISATION OF TMP.

Of the drugs which fall into this category only the halogenated pyrimidines will be considered. When the pyrimidine ring is halogenated in position 5 by chlorine, bromine or iodine, the compound may be regarded primarily as a thymine analogue since the larger van der Waal's radius of these atoms more closely resembles that of a methyl group than a hydrogen. A striking property of these compounds is their capacity to replace thymine residues in DNA. Bromuracil (Weygand et al, 1951; Litman and Pardee, 1956; Dunn and Smith, 1954; Zamenhoff and Griboff, 1954), iodouracil (Dunn and Smith, 1954; Zamenhoff and Griboff, 1954), bromodeoxyuridine (Kit et al, 1958; Eidinoff et al, 1959b), iododeoxyuridine (Prusoff 1959b) and chlorouracil (Dunn 1957) have all

been shown to be extensively incorporated into DNA of bacterial or mammalian systems in some instances replacing 50-100 per cent of the thymine component (Hacker et al, 1954; Prusoff et al, 1953).

HALOGENATED URACILS.

Chloro-, bromo-, and iodo- uracil inhibit the growth of microorganisms (Mitchings et al, 1945; Dunn and Smith, 1957). Under certain conditions, however, they may actually stimulate growth (Mitchings et al, 1945; and 1948; Prusoff, 1954) and bromodeoxyuridine can substitue for thymidine for short periods in the reproduction of Hela cells in culture under conditions in which thymidine is normally required (Makala, 1958). In so far as they have been investigated, the halogenated uracils are much less active in mammalian systems than the corresponding nucleoside derivatives and consequently are poor inhibitors of tumour growth (Kit et al, 1958; Eidinoff et al, 1957 and 1959b; Prusoff et al, 1953).

HALOGENATED RIBOSIDES OF URACIL.

Chlor-, bromo- and iodo- uridine have all been synthesised but they have not been found to possess striking biological activity (Kit et al, 1958; Eidinoff et al, 1957 and 1959b). The utilisation of iodouridine by normal tissues, regenerating rat liver and experimental tumours in vivo has been investigated (Prusoff, 1953) but significant accumulation of the compound in these tissues did not occur. There was, however, a relatively high concentration of iodine in the thyroid gland indicating extensive metabolic degradation of the analogue.

HALOGENATED DEOXYRIBOSIDES OF URACIL.

Bromodeoxyuridine and Chlordeoxyuridine.

Both these compounds can replace 30-60 per cent of the

DNA-thymine of human cells grown in culture without loss of viability (Djordjevic and Szybalski, 1960; Szybalski and Djordjevic, 1959). This incorporation can be facilitated by inhibition of the thymidylic synthetase reaction by the addition of fluorodeoxyuridine to the medium. Under these conditions the further addition of bromodeoxyuridine stimulates the reproduction of the cells and gives rise to further incorporation of the analogue. When both strands of DNA have a sufficiently large proportion of the methyl groups of their thymidylic acid content replaced with bromine the threshold of radiation sensitivity is thought to be reduced (Djordjevic and Szybalski, 1960). (see below).

Bromodeoxyuridine does not possess striking antitumour activity when used without combination with radiation (Jaffe and Prusoff) but a moderate inhibition of tumour cell reproductive capacity of mouse leukaemia cells has been reported (Berry and Andrews, 1961). It appears to have little or no toxicity for mice or humans (Holmes et al, 1954).

Iododeoxyuridine (IUdR).

Thymidine is an efficient precursor of DNA-thymine (Reichard and Estborn, 1951; Plentl and Schoenheimer, 1944) but the other methylated uracil derivatives such as thymine (Plentl and Schoenheimer, 1944; Brown et al, 1952; Holmes et al, 1954) and 5-methylorotic acid (Holmes and Prusoff, 1954) and 5-methyluridine (Friedkin and Roberts, 1954) are not. It is therefore not surprising that the corresponding iodinated derivatives (iodouracil, iodoorotic acid and iodouridine) did not inhibit the biosynthesis of DNA-thymine or that they were not

incorporated into DNA (Prusoff, 1954 and 1960a). However, since thymidine is an efficient precursor of DNA-thymine the corresponding analogue (iododeoxyuridine, (IUdR) might be expected to fulfil both these functions.

Prusoff first synthesised IUdR in 1959c. When deoxyuridine, iodine, chloroform and nitric acid were refluxed gently together white needle crystals of IUdR were formed. As expected it was found that this compound inhibited the utilisation of radioactive thymidine for the biosynthesis of DNA-thymine in Ehrlich ascites carcinoma cells in vitro (Prusoff, 1960a). Moreover, IUdR also inhibited the incorporation of ^{14}C-orotate and ^{14}C-formate into DNA-thymine but not of ^{14}C-orotate into the cytosine of DNA or into the cytosine and uracil of RNA (Prusoff, 1960). In contrast to fluorodeoxyuridine (Cohen et al, 1958; Harbers et al, 1959), IUdR appeared to inhibit the utilisation of TMP rather than its formation (Prusoff, 1960a; Kit et al, 1958). Moreover, by growing mammalian cells in the presence of radioactive IUdR and then examining their DNA it can be shown that a large proportion of DNA-thymine may be replaced by the iodinated analogue (Prusoff, 1959b and 1960b; Mathias and Fischer, 1959a). For example, after a single reproduction of mouse lymphoma L5178Y cells in culture, in the presence of ^{131}I-UdR approximately one third of the TMP component of DNA was replaced by iododeoxyuridylic acid (Mathias et al, 1959b). Similar results have been obtained in vivo when IUdR has been administered to mice bearing Ehrlich ascites tumour cells (Prusoff, 1959c). Under these circumstances, however, thymidine was utilised preferentially to a fortyfold greater extent

than IUdR.

(a) Antitumour activity in rodents.

IUdR was found to inhibit the rate of growth of several experimental tumours by 50 per cent or more as compared to the growth rate in untreated control animals (Jaffe and Prusoff, 1960). The drug was administered by intraperitoneal injection in a dose of 100 to 150 mg./kg. body weight and in this dosage did not produce significant host toxicity. In a similar dosage it was, however, found to be ineffective on the growth of Walker carcinosarcoma 256 in young rats. Only additive effects on the growth of lymphoma 1210 were seen when IUdR was administered to mice in combination with azathymine, azauridine, fluorodeoxyuridine or amethopterin. The combination of IUdR and 6-mercaptopurine for reasons not understood produced less than additive effects.

(b) In vivo metabolism of IUdR.

The metabolism of IUdR labelled with ^{131}I or ^{3}H appears to involve the cleavage of the deoxyribose moiety with the formation of iodouracil which is subsequently dehalogenated (Prusoff et al, 1960c) to form uracil and inorganic iodide. Direct deiodination of IUdR has not been observed. The breakdown of IUdR to uracil and iodide occurs very rapidly, some of the iodine being utilised to iodinate the proteins of the tissue. 74 per cent of the radioactivity of an intraperitoneal dose of ^{131}I-UdR in mice appeared in the urine within 4 hours and 91 per cent with 24 hours (Prusoff et al, 1960c). About 83 per cent of the radioactivity was present as free iodide, the remainder being IUdR, iodouracil and several other unidentified

substances.

(c) Toxicity.

In Swiss mice the LD50 of IUdR was 2.5 g./kg. when administered in a single dose. The LD50 upon repeated daily administration of the drug was 312 mg./kg. The toxicity could be completely prevented by prior administration of thymidine (Prusoff et al, 1960c)

Doses of 100 mg./kg. to dogs for 10-20 days produced anorexia, leucopenia, thrombocytopaenia and occult blood in the stools. There was inhibition of cellular proliferation in the blood forming tissues and in the intestinal epithelium (Welch et al, 1960).

Iododeoxycytidine (ICdR).

The halogenated derivatives of deoxycytidine have been investigated in the hope that they might have greater metabolic stability, which in turn might result in their more extensive incorporation into the acid soluble nucleotide pools as well as into DNA. There is evidence to suggest that the amino group in lieu of oxygen in position 6 of the pyrimidine ring results in greater metabolic stability. The cytosine containing nucleosides, unlike those of thymine and uracil, do not participate in certain enzyme-catalysed reactions which lead to displacement of their sugar component (Verdier and Potter, 1960). The enzyme deoxycytidylate deaminase has been found to be present in rapidly growing mammalian tissues such as neoplasms, embryonic tissue, regenerating liver, thymus and bone marrow but has been reported to be absent in most normal tissues. Recently, however, high concentrations of the enzyme have been found in the kidney (Creasy, 1962).

Iododeoxycytidine was first synthesised in 1961 (Chang and Welch, 1961b) and so far has been investigated only slightly. As predicted, however, it acts in the same way as IUdR but is is catabolised surprisingly quickly (Cramer et al, 1962). Following the injection of mice with ^{3}H-iododeoxycytidine, chromatography of the urine showed the drug to be rapidly and extensively metabolised. It is, however, less toxic to mice than IUdR and this together with its increased solubility in water and resistance to thermal decomposition in solution offer the possibility of significant advances over IUdR in the treatment of neoplastic disease.

Bromodeoxycytidine.

Bromodeoxycytidine was also synthesised (Chang and Welch, 1961a) because of the possibility of its greater metabolic stability. It has been shown to be resistant to nucleosidases (Cramer et al, 1961) *in vitro* and moreover in mice and rats *in vivo* the bromide appears to be cleaved from it less readily than from bromodeoxyuridine (Kriss et al, 1961). Tritiated bromodeoxycytidine has been shown to be incorporated into DNA as 5-bromo-2'-deoxyuridylic acid replacing an equivalent amount of TMP. Whether phosphorylation precedes or follows deamination has not been determined but the metabolic conversion of bromodeoxycytidine to the deaminated derivatives has been observed both in a cell free system and in intact neoplastic murine mast cells (P875Y). When administered in rats, ^{82}Br-bromodeoxycytidine is distributed differently through the body from ^{82}Br-bromodeoxyuridine. This may be due to the concentration of deoxycytidylate activity in certain tissues such as the bone marrow.

Unfortunately, rapid debromination takes place in man following intravenous administration of bromodeoxycytidine (Welch, 1961) and in this respect the drug offers no significant advantage over the corresponding uracil derivatives.

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CHAPTER III.

CLINICAL STUDIES WITH THE HALOGENATED PYRIMIDINES.

Extensive clinical trials have been carried out with fluorouracil and initial studies have been made with fluorodeoxyuridine, iododeoxyuridine and bromodeoxyuridine.

5-Fluorouracil and 5-Fluorodeoxyuridine.

Over 3,000 cases of advanced neoplastic disease have been treated with fluorouracil and fluorodeoxyuridine in various centres mainly in the United States of America and trials are now beginning in the United Kingdom. Both drugs have been found to be of value in various forms of advanced carcinoma but particularly in carcinoma of the breast and bowel (Ansfield and Curreri, 1959 and 1960; Young et al, 1960; Hurley and Hall, 1960; Vaitkevicius et al, 1961a; Weiss and Jackson, 1961; Field, 1960) and encouraging results have been obtained in the treatment of carcinoma of the oropharynx, stomach, uterine cervix, ovary, urinary bladder and hepatoma (Young et al, 1960; Vaitkevicius et al, (1961a and b); Weiss and Jackson, 1961; Field, 1960; Calabresi et al, 1960; Cornell et al, 1960; Staley et al, 1961; Deren and Wilson, 1960; Wilson, 1960). The compounds are not very effective against either acute or chronic leukaemia (Curreri et al, 1958; Wolman and Gens, 1959; Hartman et al, 1960) or against malignant melanoma, hypernephroma, carcinoma of lung, pancreas, prostate or oesophagus (Staley et al, 1961; Curreri et al, 1958; Hurley and Hall, 1960; Corle, 1960; Ferguson and Humphrey, 1960). Preliminary results indicate (Curreri and Ansfield, 1962) that fluorodeoxyuridine in good risk patients has a superior therapeutic

index to fluorouracil, and that it is superior as a palliative agent in the treatment of cancer of the breast, colon and rectum. It is, however, equally toxic and associated with a very narrow therapeutic index in poor risk patients. When fluorodeoxyuridine is administered in a dose of 3mg./Kg./day by continuous I.V. infusion it is said to produce equal toxicity to that of 30mg./Kg./day given (Sullivan et al, 1960) by rapid daily injections over a similar period. Further work has demonstrated that while this may be so the later dosage regimen produces a much greater clinical response suggesting that the method of administration greatly influences the site of drug action (Ansfield et al, 1962).

Although regression of the tumour occurs in certain circumstances there is no case on record of complete disappearance and there is no conclusive evidence of actual prolongation of life (Hurley and Hall, 1960; Corle, 1960; Ellison, 1961).

The degree of improvement following the administration of a drug to a patient suffering from advanced neoplastic disease is always difficult to assess and in an attempt to put this on a more scientific basis Ansfield and Curreri (1959) have laid down certain criteria of response to either fluorouracil or fluorodeoxyuridine which are probably applicable to other drugs of this category. They are as follows:- (1) a measurable reduction in tumour size, (2) general symptomatic improvement, (3) improved physical performance, (4) gain or maintenance of body weight and (5) persistence of all these features for at least 2 months. Other criteria have also been recommended (Ansfield and Curreri, 1959; Curreri et al, 1958) by the same workers

for the selection of patients for treatment and they are as follows:- (1) good nutritional state, (2) no major surgery for at least 30 days prior to therapy, (3) adequate bone marrow function i.e. absence of evidence of marrow infiltration by tumour tissue and no previous therapy with X-rays or ankylating agents and (4) good renal function. Patients who fulfil these criteria are treated with 15 mg. of fluorouracil per Kg. by rapid intravenous infusion daily for 5 successive days. If there are no toxic manifestations within the next 48 hours, the drug is given in half that dose on alternate days to a maximum of 4 doses. If the patient does not fulfil these criteria he is given a modified course of the drug which consists of 15 mg./Kg. for 3 successive days followed by a dose of 75 mg./Kg. on the fifth day (Ansfield and Curreri, 1959; Curreri et al, 1958). Fluorodeoxyuridine is administered in exactly the same way but in twice the dose (in mgm.) used for fluorouracil.

The toxic effects of both compounds are seen mainly in the bone marrow and gastrointestinal tract (Young et al, 1960; Ansfield and Curreri, 1960; Vaitkevicius et al, 1961; Calabresi et al, 1960; Curreri et al, 1958; Moore and Koike, 1960; Ferguson and Humphrey, 1960). The patient may first complain of anorexia and nausea and these symptoms are shortly followed by stomatitis and diarrhoea. The stomatitis may proceed to ulceration and necrosis (Calabresi et al, 1960) and similar lesions have been seen in the colonic mucosa at post mortem (Gold et al, 1960). Bone marrow changes take the form of an increase in the myeloid erythroid ratio (Vaitkevicius et al, 1961) with megaloblastic change in the red cell series in some

instances (Brennan et al, 1960). These changes progress to hypoplasia or aplasia of the bone marrow with a pancytopaenia in the peripheral blood. Leucopaenia is usually most marked between the 9th and 11th days after the commencement of therapy but may occur anywhere between the 5th and 21st day (Vaitkevicius et al, 1961; Curreri et al, 1958). It is likely to be particularly severe in patients who have previously been treated with alkylating agents and radiotherapy to the spine or pelvis (Young et al, 1960; Vaitkevicius et al, 1961, Calabresi et al, 1960; Curreri et al, 1958).

Another side effect of these drugs is hair loss, which is usually slight but may progress to total alopaecia (Vaitkevicius et al, 1961, Curreri et al, 1958). Other epidermal structures such as skin and nails may also be affected giving rise to dermatitis, pigmentation of the skin and ridged nails (Vaitkevicius et al, 1961, Curreri et al, 1958). Neurological changes have been reported (Staley et al, 1961) and myelopathy following intrathecal administration was seen in one case (Koenig, 1959).

Bromodeoxyuridine.

This compound has little or no effect on malignant or normal tissue when administered to man but preliminary studies have suggested that its incorporation into the DNA of malignant cells may lower their threshold of radiation sensitivity (Brennan et al, 1960).

Iododeoxyuridine. (IUdR).

Preliminary clinical studies with IUdR have been reported (Welch and Prusoff, 1961; Calabresi et al, 1961). Patients with advanced neoplastic disease not amenable to surgery were treated with the compound and significant tumour inhibition was noted in some

patients with malignant melanoma, fibrosarcoma, epithelioma of the tongue and mucoepidermoid carcinoma of the parotid gland. Only modest tumour inhibition was obtained and this was accompanied by significant toxicity.

The compound was administered intravenously. IUdR is a weakly acidic compound which is poorly soluble in alkaline solution and which is partially decomposed by autoclaving. It was, therefore, prepared for intravenous administration by sterile filtration of a solution of 5 per cent glucose pH 8.6 containing 6 mg. of IUdR/ml. Daily doses of 17 to 124mg./kg. were administered over a period of 1 to 3 hours. A total dose of 60 to 720 mg. was given over a period of 2 to 7 days. The same workers are at present experimenting with different dosage regimens infused over longer periods of time, thus making the drug available for cellular metabolism during an increased number of reproductive cycles.

Following the infusion of ^{131}I-UdR in doses of 80mg./kg. and 100 mg./kg. respectively in two patients, the level of radioactivity in the blood progressively fell reaching 50% of the five minute level in 7 to 10 hours. Twenty four hours after the cessation of the infusion 88 per cent of the radioactivity was removed in the urine. Urine collected during the infusion contained about 50 per cent of its radioactivity as iodide and chromatography of the urine demonstrated that the IUdR was undergoing rapid destruction in the human subject with the formation of both iodouracil and iodide.

No immediate toxic effects have been noted in man except for mild iodism which consisted of oedema of the mucous membranes, ptyalism and acneform dermatitis. Delayed toxic effects are almost identical

to those seen with fluorouracil and fluorodeoxyuridine but the alopaecia tends to be more severe. The three effects most easily observed are leucopaenia, stomatitis and alopaecia and in patients receiving over 600mg./kg. body weight all three were invariably present. Anorexia, diarrhoea, nausea and vomiting occasionally occur but are less common than with fluorouracil. Thrombocytopaenia and nail changes are occasionally seen. Some of the undesirable side effects can be prevented by the use of thymidine. Calabresi (1961) recently treated 4 patients with an infusion of thymidine (4 mg./kg.) into one external carotid artery for about 4 hours beginning about 15 minutes before a 2 hour intravenous infusion of IUdR in a dose of 120 mg./kg. This was carried out on 5 successive days. The drug produced a leucopaenia but stomatitis was prevented completely and hair loss was prevented on the infused side only. Further studies are in progress in an attempt to provide regional protection to the bone marrow. Such procedures may eventually enable larger doses of IUdR to be administered to the patient and therefore to the tumour without the production of serious side effects.

IUdR inhibits the <u>utilisation</u> of TMP and fluorodeoxyuridine inhibits the <u>formation</u> of TMP. It was therefore thought that these drugs might act synergystically. Studies undertaken by Young et al, (1960) showed marked potentiation of IUdR by the addition of fluorodeoxyuridine but since toxicity to normal tissues was increased to an equal degree, it is doubtful if this observed synergism will be of any advantage clinically.

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CHAPTER IV.

EFFECTS OF THE HALOGENATED PYRIMIDINES ON THE THRESHOLD OF RADIATION SENSITIVITY.

INTRODUCTION.

One of the major obstacles in the therapeutic measures aimed at destroying tumour tissue is that in many instances it is impossible to selectively destroy the neoplasm without at the same time producing considerable injury to the normal tissues; that is to say the therapeutic ratio, which is the ratio of the effect on normal tissue to that on tumour tissue, is low. The tolerance of normal tissues to either chemotherapy or radiotherapy therefore usually becomes the limiting factor in the total dose of drug or X-rays that it is permissible to administer. In recent years with the advent of new forms of radiotherapy it has been possible to direct ionising radiations to strictly localised areas with very little scatter to the normal tissues. Sooner or later, however, the limit of tolerance is reached by the overlying tissues and this form of therapy has to be abandoned. If the tumour tissue could selectively be made more sensitive to ionising radiation while the overlying tissues remained unchanged the effective dose of radiation that could be administered would be considerably increased. Moreover, by lowering the threshold of radiation sensitivity in the tumour tissue, certain neoplasms that are usually considered resistant to irradiation might become amenable to such therapy.

One possible approach to the solution of this problem is the combined use of chemotherapeutic agents with radiotherapeutic measures and prominent in studies in this connection are the halogenated pyrimidines. The effect of combined therapy with antimetabolites and x-irradiation may be additive or it may be synergistic so that the combined effect is greater than the simple addition effect of each agent. The aim of therapy is to achieve a synergistic effect and/or an increase in the therapeutic ratio. If the two agents are synergistic and also produce an increase in the therapeutic ratio then therapeutic synergism is said to exist.

IN VITRO STUDIES:

Using a thymine deficient strain of E.coli Greer (1960) demonstrated that if the organisms were allowed to grow in the presence of 5-bromuracil with consequent replacement of a considerable portion of the thymine in the DNA by the analogue then their sensitivity to the ultraviolet irradiation was markedly increased. Szybalski and his associates (Djordjevic and Szybalski, 1960; Szybalski and Djordjevic, 1959; Erikson and Szybalski, 1961) with cultures of human cell lines have shown that incorporation of iodo-, bromo- or chlordeoxyuridine into the DNA increases the sensitivity of the cell to ultraviolet light and x-irradiation by anything up to 22 times. The chlorine and bromine compounds may be incorporated into DNA with replacement of up to 30 to 60 per cent of the DNA-thymine without loss of viability of the cells provided such replacement is achieved gradually during many successive reproductive cycles.

Greater incorporation of the preformed thymidine analogues could be obtained if de novo synthesis of TMP was blocked by the addition of fluorodeoxyuridine to the medium. These authors attributed the greater viability of the cells in the presence of bromo- and chlorodeoxyuridine than in the presence of IUdR to the fact that van der Waal's radius of the methyl group more nearly approached that of the bromine and chlorine than it does that of the iodine atom.

The chlorine compound was less effective than the other two in lowering the threshold of sensitivity both to ultraviolet light and to x-irradiation. IUdR brought about the greatest reduction in the threshold of sensitivity to X-rays but bromodeoxyuridine was more effective when ultraviolet light was employed (Erikson and Szybalski, 1961).

After many replications had occurred in the presence of these analogues a large proportion of TMP in both strands of the double helix of DNA had been replaced. This "bifilar" labelling, the authors believed, produced in the cells a reduction in the threshold of radiation sensitivity after exposure either to ultraviolet light or to x-irradiation. Moreover, they suggested that the compound must be present in both strands of the DNA since if such radiosensitised cells were allowed to undergo a single replication in the absence of the analogue restoration of their former relative resistance to radiation injury was observed (Djordjevic and Szybalski, 1960). Other workers using Chinese hamster (Mohler and Elkind, 1961) or human bone marrow (Humphrey et

al, 1961) cells have also found potentiation of radiation sensitivity following exposure to bromodeoxyuridine.

The question arises as to whether sensitisation might be dependent upon the presence of phosphorylated derivatives of the analogue in the cytoplasm rather than in the DNA and the fact that one reproductive cycle in the absence of the analogue restored resistance to radiation injury does not in itself preclude this possibility since this would also most probably lead to a marked diminution in the halogenated nucleotides contained within the cytoplasm. Szybalski and Opara-Kubinska (1961) have produced some evidence to show that DNA is the principal determinant of cell radiosensitivity, at least in bacterial cells. They grew Bacillus subtilis organisms in the presence or absence of bromodeoxyuridine and found that the incorporation of the analogue resulted in similar increments of radiation lethality of intact cells and of decreased inability of DNA extracted from these cells to transform indole-requiring cells to prototrophy. These experiments suggest that in intact bacterial (and mammalian by close analogy) cells DNA is the principal and indispensable target of lethal ultraviolet and X-ray effects. Radiation effects on other cellular activities such as observed inhibition of new enzyme (protein) synthesis would thus seem to represent only secondary expressions of radiation damage to DNA structure and biological function. Kaplan et al (1962) have also carried out bacterial studies indicating that the radiosensitivity conferred by the halogenated pyrimidines is dependent on their incorporation into the

DNA. They showed, for example, (as did Djordjevic and Szybalski, 1960) that incorporation of bromocdeoxyuridine into DNA was progressive with time and the degree of radiosensitisation also increased progressively in a similar manner to a plateau at about 40 to 50 per cent substitution of bromuracil for thymine. Other analogues which were not incorporated into DNA lacked radiosensitising activity. These authors, however, did not find absence of radiosensitisation with unifilar labelling of DNA but found it to be 50 per cent of that seen when both strands were labelled. They were unable to offer any definitive explanation of the mode of action of the halogenated derivatives in this respect but DNA-containing bromodeoxyuridylic acid has been shown to have a slightly higher melting temperature than its normal counterpart (Kit and Hsu, 1961).

The effect of the halogen is presumed to be either in the strengthening of the hydrogen bonding with adenine or in changing the interaction of DNA with cation since the melting temperature is a function of cation concentration. As Lajtha (1960) points out most primary lethal actions of radiations are due to inhibition of mitosis, chromosome damage, or gene mutations. It would therefore seem reasonable to examine chromosome morphology of "radiosensitised" cells. Djordjevic and Szybalski (1960) found that chromosome morphology was not significantly altered after incubation of the cells with levels of bromodeoxyuridine which conferred radiation sensitivity; only at very high levels which noticeably affected cell viability was chromosome damage noted. Humphrey et al (1961), however, cultured an <u>in vitro</u> strain (LET-1) of Lettré mouse ascites tumour

and L-P59 cells in the presence of low concentrations of bromodeoxyuridine (5 and 10 ug/ml.) for periods of time varying from 6 to 96 hours. These cells were then subjected to x-irradiation and chromosomal damage observed at metaphase and anaphase at various times after irradiation. The observed changes indicated that the cells were approximately twice as radiosensitive as that of the control cells. The maximum increase in sensitivity was observed when the incubation period was 24 hours with LET-1 cells and 48 hours with the L-P59 cells prior to irradiation.

IN VIVO STUDIES:

Kriss and Revez (1962) have demonstrated a greater inhibition of post treatment growth curves of Ehrlich ascites cells in vivo when X-rays were administered following a course of bromodeoxyuridine than when X-rays were administered alone. Berry and Andrews (1961) treated mice with the ascites form of mouse transplantable leukaemia with IUdR or bromodeoxyuridine and/or x-irradiation. At doses of 300 mg./kg./day for 4 days IUdR alone did not affect the number of leukaemic cells required to produce a 50 per cent take in a group of recipient mice of the same strain; but this dose markedly enhanced the radiation effects i.e. brought about a marked reduction in the number of cells reproductively intact after a single dose of X-ray. The amount of radiation potentiation was proportional to the dose of IUdR administered. Bromodeoxyuridine alone produced a modest inhibition of tumour cell reproductive capacity but also potentiated the radiation effects at low dosage levels of the drug.

CLINICAL STUDIES:

Kligerman (1960) reported the results of combined IUdR therapy with irradiation on humans carried out at Yale University. Patients were treated for 5 or 6 days successively in a dose of 100-120 mg./kg. over a period of several hours. Of the nine patients treated with IUdR and radiotherapy 6 were available for evaluation and of these, 2 were thought to have shown definite potentiation to X-ray therapy. The same author also reported on twenty patients that were treated with fluorouracil in combination with radiotherapy and in general these patients did not show regression superior to that seen with X-rays alone but were superior to the drug alone. There was, however, one exception; a patient with multiple cervical and supra-clavicular nodes secondary to buccal carcinoma had one node treated with 2,500 rads in 5 days and a second node was treated with fluorouracil and X-rays simultaneously. Marked regression of the latter node was seen but the one receiving X-rays alone did not change. Foye et al (1960) reported rapid and significant regression in 12 out of 18 patients receiving a combination of X-rays and fluorouracil when the X-rays were given over 12 days (2,000 rads) and the fluorouracil given in a dose of 90mg./kg. over the first 5 days. Three others showed moderate regression. One patient with bilateral lung metastases from an embryonal cell carcinoma of the testis had the right lung treated with combined therapy and the left lung with X-rays alone three weeks later. Only slight regression occurred in the left lung but complete regression occurred in the right lung. Vermund (1960) treated 20 patients in a random manner with

fluorouracil either alone or in combination with X-rays but he found no difference in the mean survival of the two groups. Fletcher (1960), however, found that in patients with head and neck carcinoma who had received fluorouracil, regressions took place at the same rate with X-rays in a dose of 6,000 rads in 6 weeks as when X-rays in a dose of 6,000 rads in 4 weeks were given to patients who had received X-rays alone. Krant et al,(1961) reported the results in humans with various "radioresistant" tumours when given bromodeoxyuridine alone in a dose of 15 mg./kg./day over a period of 4-10 days and followed by irradiation of the tumour. No toxicity save for a metallic taste was noted. When possible, prior to or after combination therapy radiation therapy alone was given in order to compare the results in the same patients. In 4 patients so treated definite responses "probably attributable to sensitisation" were observed in three (one patient with a carcinoma of the stomach, and two with carcinoma of the lung). The fourth patient with a testicular tumour responded to X-ray therapy alone given later as well as he did to the combination therapy.

DISCUSSION:

The radiosensitising effect of the halogenated pyrimidines and also certain other drugs such as actinomycin D., hormones and alkylating agents opens up new possibilities for selective potentiation of the radiotherapy of localised tumours. The halogenated pyrimidines acting as thymine or thymidine analogues are of particular value in this respect for owing to their utilisation for the synthesis of new DNA, they have a selectivity of action for dividing cells.

With the advent of new devices for the delivery of strictly localised irradiation it then becomes possible to cause regression of the tumour without interfering with actively dividing normal cells in other areas of the body such as the bone marrow and gastrointestinal tract.

Much more work needs to be done for the proper evaluation of these compounds as radiosensitising agents in man. Many of the cases treated with combination therapy have been inadequately controlled. Each patient should serve as his own control whenever possible and patients with such lesions as bilateral pulmonary carcinoma or multiple metastatic skin nodules or lymph nodes are most suitable. In such patients a course of the drug may be administered systemically and selected tumours irradiated afterwards and compared with similar non-irradiated lesions in the same patient. If no such lesions are present the following steps should be taken. (1) Irradiation of the lesion followed by an observation period of sufficient length for the full effects of radiation to be noted (e.g. one month). (2) A course of the drug in question may then be given and a further period of time allowed to elapse for the evaluation of drug therapy alone. (3) This can finally be followed by combination therapy and a comparison made with results achieved by either treatment alone.

Another possible approach to this problem is the implantation of tissue from the primary tumour in multiple sites under the skin of the arms so that multiple subcutaneous nodules of comparable nature develop. These can then be treated with combinations of therapy in

varying dosages with certain nodules serving as controls. This procedure, however, raises ethical as well as considerable technical problems. More work is required also to determine which techniques of administration of the drug will facilitate its incorporation into the DNA of the neoplastic cell.

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CHAPTER V.

EXPERIMENTAL PROCEDURE.

INTRODUCTION.

A brief account of the aims of the investigations that were undertaken and the methods employed is given below.

SECTION I. IUdR had been shown previously to inhibit the synthesis of DNA-thymine (Prusoff, 1959c and 1960). Experiments were, therefore, undertaken to determine at which point or points on the biosynthetic pathway IUdR exerted its inhibition. Cells from various sources were incubated with either tritiated thymidine (^{3}H-thymidine) or carbon labelled formate (^{14}C-formate) in the presence of IUdR. The thymine nucleotide fractions were then extracted and separated by means of chromatography. The total radioactivity in each fraction was determined and compared with that in control cells in which no IUdR had been added. The specific activity of DNA-thymine was also determined and compared with that in control cells in a similar manner.

SECTION II. The inhibitory effects of IUdR and ICdR on the biosynthesis of thymine nucleotides and DNA-thymine were compared using similar techniques to those in Section I. In addition, their relative ability to inhibit the growth of micro-organisms and solid tumours in animals was also determined.

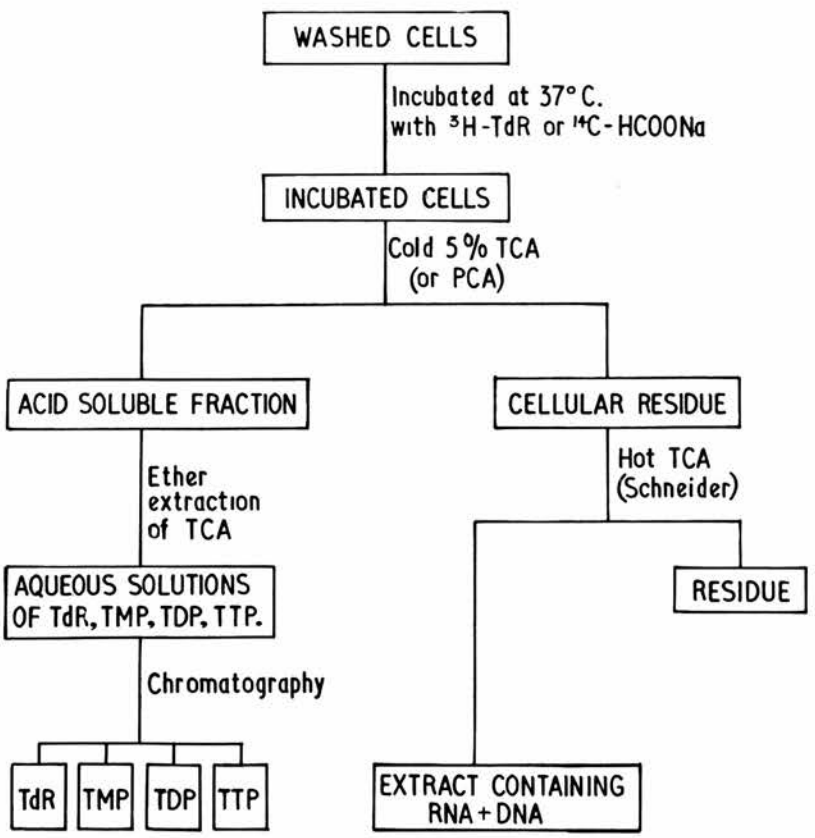
SECTION III. The ability of deoxycytidine to increase the rate of biosynthesis of the thymine nucleotides and DNA-thymine was investigated over periods of time ranging from 0.5 min. to 240 mins.

Cells were incubated with ^{14}C-formate and the radioactivity in the various fractions analysed using the same techniques as in Section I. The ability of deoxycytidine to reduce the inhibitory effects of IUdR was also studied.

In figure 4 is shown the overall experimental procedure that was adopted for the incorporation of radioactive thymidine or formate into the phosphorylated derivatives of thymidine and DNA-thymine of mammalian cells and the subsequent isolation and separation of these various fractions.

Figure 4.

DIAGRAM OF EXPERIMENTAL PROCEDURE FOR ISOLATION AND SEPARATION OF THE PHOSPHORYLATED DERIVATIVES OF THYMIDINE AND DNA-THYMINE



MATERIALS.

STUDIES WITH MAMMALIAN CELLS.

Nutrient Buffer Solutions:

One of two nutrient buffer solutions was employed and both were found to be equally satisfactory.

(a) Totter's Modification of Chambers Solution. (Totter, J.R., 1954; (Chambers, R., 1943).

A solution of the following composition was prepared.

Potassium Chloride	2.6 g.
Sodium Chloride	0.78 g.
Sodium citrate	1.25 g.
Glucose	5.00 g.
Dihydrogen potassium phosphate	0.48 g.

The pH was adjusted to 7.5 and the volume made up to 100 ml. with distilled water.

This solution was diluted 5 times before use.

(b) Krebs III Buffer Solution. (Krebs, H.A., 1950).

This solution was prepared as follows:-

0.9% Sodium chloride	95 parts
1.15% Potassium chloride	4 "
0.11 M calcium chloride	3 "
2.11% Potassium dihydrogen phosphate	1 part
3.82% Magnesium sulphate ($Mg.SO_4\ 7\ H_2O$)	1 part
1.3% Sodium bicarbonate	3 parts
Sodium phosphate buffer (100 parts 1.78% Na_2HPO_4 $2\ H_2O$ + 25 parts 1.38% $Na\ H_2PO_4\ H_2O$)	3 parts

0.16 M Sodium pyruvate	4 parts
0.1 M Sodium fumarate	7 "
0.16 M Sodium-L-glutamate	4 "
0.3 M (5.4%) glucose	5 "

The pH was adjusted to 7.2

Both solutions were stored at -10°C until required for use.

Radioactive Materials:

The experiments were carried out using one of the following radioactive materials.

(a) Sodium ^{14}C-formate with a specific activity of 3 μc per μM/ml.

(b) ^{3}H-thymidine with a specific activity of 50 μc per μM/ml.

Antimetabolites:

Iododeoxyuridine or iododeoxycytidine was dissolved in Krebs III buffer solution (pH 7.2) or in water and the pH adjusted to 7.5. The final concentration of the solution used was 5 μM/ml.

Deoxycytidine:

This metabolite was employed in some experiments. The solution was at a concentration of 100 μM/ml.

STUDIES WITH MICROORGANISMS.

Growth Medium for Streptococcus Faecalis (ATCC 8043).
Double Strength.

Dextrose anhydrous	40.0 g.
Na_2 citrate $2H_{20}$	63.0 g.
K_2HPO_4 anhydrous	6.0 g.
Acid hydrolysed casein	1.0 g.
D-L Trytophane	800 mg.

Adenine sulphate	50 mg.
Guanine hydrochloride	5.0 mg.
Xanthine (monosodium salt)	200 mg.
L-Asparagine	600 mg.
L-cystine	500 mg.
Vitamin Mixture (see below)	100 ml.
Glutathione	5.0 mg.
$MnSO_4 H_2O$	200 mg.
Salt solution (see below)	10.0 ml.

The final pH was adjusted to 6.8 and distilled water added to 500 ml.

Vitamin Mixture (Double strength).

1) Pyridoxine hydrochloride 4.0 mg.
   Thymine hydrochloride 4.0 mg.
   Calcium hydrochloride 8.0 mg.
   Nicotinic acid 8.0 mg.
   Dissolve in 200 ml. distilled water. Add 200 mg. biotin.
2) 10 mg. riboflavine dissolved in 200 ml. 0.02 M. acetic acid.
3) Add 40 ml. sodium acetate buffer solution (pH 4.5).
4) Adjust volume to 1 litre with distilled water.

Acetate Buffer pH 4.5.

Glacial acetic acid	18.75g(19.8 ml.)
Sodium acetate	38.65 g.

Adjust volume to 500 ml. with distilled water.

Salt Solution.

$MgSO_4 H_2O$	1.5 g.
NaCl	1.0 g.

$FeSO_4\ 7\ H_{20}$ 1.0 g.

$MnSO_4\ H_{20}$ 750 mg.

Adjust pH to 6.8. Make up volume to 500 ml. with distilled water.

To the medium folic acid, thymidine or thymine was added in a concentration sufficient to allow half maximum growth of the organism i.e. 1.8×10^{-6} µm/ml. 0.004 µm/ml. and 0.004 µm/ml. respectively.

METHODS.

STUDIES WITH MAMMALIAN CELLS.

Preparations of cell suspensions.

All cell suspensions from animals were prepared in the cold. White cells from human leukaemic blood were separated at room temperature.

(a) Mouse Tumours.

1. The Ehrlich Ascites Tumour was maintained in DBA/2 mice, 5 or 6 days following the intraperitoneal innoculation of mice with tumour cells, ascitic fluid was collected from a suitable number of mice and pooled in a graduated centrifuge tube (40 ml.) which contained a few ml. of the modified Chambers or Krebs III nutrient buffer solution. After centrifugation the cells were washed once in normal (0.9 per cent) saline and then resuspended in Chambers or Krebs solution to form a 25 per cent suspension.

2. L-5178Y lymphoma.

The mice bearing the lymphoblastic leukaemia L-5178Y were ADR x DBA/2 F1 Hybrids: The Y strain of leukaemia L-5178 was developed by G.A. Fischer and J.J. Jaffe from a single cell, isolated

in vitro and grown initially in a medium which has been described by G.A. Fischer and A.D. Welch (1957). These cells were prepared in the same way as the Ehrlich ascites cells.

(b) The Walker Rat Tumours.

Walker carcinosarcoma 256 was implanted subcutaneously in male weanling albino rats. After 10 days the tumours were removed and homogenised in Krebs III buffer. After centrifugation the cells were resuspended in buffer to form a 25 per cent suspension.

(c) Calf Thymus.

Fresh normal calf thymus tissue was homogenised in Krebs III buffer solution and a 25 per cent suspension made as described above.

(d) Human Leukaemias.

Preparations of cells from human leukaemias were made according to the method described by Walford et al (1957). Whole blood was mixed with 25 per cent of its own volume of 6 per cent dextran solution and allowed to stand at an angle of 40 degrees at room temperature for 40 minutes. The white cell layer was then pipetted from the top and after centrifugation the cells were washed with 0.9 per cent saline and a 25 per cent suspension of cells in Krebs III buffer solution was prepared.

Incubations.

(a) Effect of IUdR or ICdR on the utilisation of ^{3}H-thymidine or ^{14}C-formate for the biosynthesis of the phosphorylated derivatives of thymidine and DNA-thymine.

Incubation mixture consisted of 1 ml. of 25 per cent suspension of cells, horse serum (0.2 ml.) iododeoxyuridine or

iododeoxycytidine in a concentration of 5 μM/ml. or 0.5 μM/ml. (1 ml.) ^{3}H-thymidine (0.1 ml.) or ^{14}C-formate (0.1 ml.) and Totter's modification of Chambers solution or Krebs III buffer solution to 2.3 ml. The control incubation mixtures contained no antimetabolite but were replaced by an equal volume of the appropriate buffer solution.

The incubations were carried out under an atmosphere of air in a Dubnoff metabolic shaker at 37°C for varying periods of time up to 2 hours after which the reaction was stopped by centrifugation in the cold. The cells were then washed twice with saline in the cold to remove most of the excess ^{14}C-formate or ^{3}H-thymidine.

(b) Effect of deoxycytidine on the utilisation of ^{14}C-formate for the biosynthesis of the phosphorylated derivatives of thymidine and DNA-thymine.

The incubation mixture (final volume 7.2 ml.) contained packed cells (0.75 ml.), horse serum (0.6 ml.), ^{14}C-formate (9 μc;3 μM) in the absence or presence of deoxycytidine (30 μM) and Krebs III buffer solution. The incubations were conducted in 50 ml. beakers under an atmosphere of air in a Dubnoff metabolic shaker at 37°C.

In the first experiment the cells were examined after 5 minutes and subsequently at 10, 15, 30, 60 and 120 minutes.

In the second experiment the cells were examined after 0.5 minutes of incubation and thereafter at doubling intervals of time up to 4 hours. The reactions were terminated by the addition of trichloracetic acid (0.5 ml., 100 per cent).

Isolation of the phosphorylated derivatives of thymidine.

Two methods were employed but the first to be described was found to be generally better.

(a) Trichloracetic Acid Extraction.

All reactions were performed at 0 to 4°C. The washed or frozen cells were suspended to ice-cold (trichloracetic acid) (5 per cent, 5-10 ml.) for 20 minutes. Following centrifugation the TCA-extraction was repeated and the supernatant fractions were combined. The TCA was removed by ether-extraction and the residual ether was removed by bubbling nitrogen through the solution. The pH was adjusted to 7.5 ± 0.5 or to 10.5 ± 0.5 with NaOH (0.2 N) depending on the method used for separation of the nucleotides (see below).

(b) Perchloric Acid Extraction.

Washed or frozen cells were suspended in 5 ml. ice-cold perchloric acid (0.5 M) for 15 minutes. After centrifugation the supernatant was decanted and adjusted to pH 9-10 with KOH (2 N) using phenolphthalein as an indicator. After centrifugation the supernatant was decanted.

Separation of thymidine and its phosphorylated derivatives.

This was attempted by a variety of procedures. During the course of this investigation one new technique was devised by the use of ion exchange paper.

(a) Column Chromatography.

Three methods were tried and each is described below.

(1) Dowex-1 formate (x 8) columns 1 cm. in diameter and 10 cm.

long were employed. They were prepared for use by washing with a solution of formic acid (6 N) and ammonium formate (2 N) followed by water. The thymidine nucleotides were adsorbed on to the column at pH 10-11 and thymidine, TMP, TDP, TTP separated by elution with formic acid (0.1 N) and ammonium formate (0.3 N, 0.6 N and 2 N successively). Thymidine, TMP and TTP (0.2 μM.) were added to the extract and each fraction was considered to be completely recovered from the column when the appropriate marker and radioactivity could no longer be detected in the eluate. Each fraction was contained in about 50-100 ml. of eluate.

(2) Dowex-1 formate (x 4) columns, 1 cm. x 25 cm. were employed. To these columns a perchloric acid extract prepared as described above was applied at pH 9-10. Gradient elution using water (500 ml.) and 1 M solution of ammonium formate (500 ml.) was carried out. After eluting with 500 ml., the upper reservoir was filled with 2 M solution of ammonium formate (pH 5, 500 ml.). The fractions were collected by means of a fraction collector and each fraction was contained in about 50-70 ml. recovered from the column after eluting with approximately 50, 285, 400 and 600 ml. of fluid with respect to thymidine, TMP, TDP and TTP respectively.

(3) DEA Cellulose Columns.

This method was described by Weissman et al (1960) and generally proved to be the most useful method and easiest to execute; 8 x 1 cm. columns were used. The cellulose was washed with 50 m. 0.5 N HCl and 125 ml. H_2O. The acid soluble extract was then applied to the columns at pH 7-8 and thymidine, TMP, TDP and TTP eluted off

successively with H_2O, 0.01 NHCl, 0.05 NHCl and 0.5 NHCl respectively. The volume of each solution required to recover all the radioactivity in each fraction was approximately 50-70 ml. The marker substances could be recovered consistently in the expected fraction and the pH of the extract when applied to the columns did not appear to be critical.

(b) A New Method using Paper Chromatography.

Thymidine and its various phosphorylated derivatives have been readily separated from each other by column chromatography (see above) (Hurlbert et al, 1954; Schneider, 1945). However, it has been difficult to obtain such clean separations using paper chromatography (Bollum and Potter, 1959). Although thymidine and TMP have been readily separated on paper, there was appreciable overlapping of TDP and TTP and the results could only be interpreted in a semiquantitative measure. Paper chromatography is often desirable because this method permits the separation of microquantities of materials and is very convenient when dealing with a large number of samples.

The method now to be described was developed during the execution of the work reported in this thesis and gives efficient separation of thymidine, TMP, TDP and TTP by paper chromatography using Whatman cellulose paper AE30. Prior to the adsorption of a solution composed of a mixture of thymidine, TMP, TDP and TTP, the paper strip was washed successively with formic acid (0.1 N), ammonium formate (0.5 N) and water for 24 hours. The first solvent, formic acid (0.01 N), was used to separate thymidine from the other

constituents. In this solvent system thymidine moved with the solvent front which was permitted to travel almost to the end of the paper. The chromatogram was allowed to dry before elution with the second solvent system, (ammonium formate 0.06 N, pH 5.0), which was allowed to flow two thirds of the distance transversed by the first solvent system. The various components were located with the aid of an ultraviolet lamp (figure 5) and readily eluted in HCl (0.1 N) with essentially 100 per cent recovery. The minimum quantity which could be located readily was 0.2 μM although smaller quantities were detectable.

Figure 5.

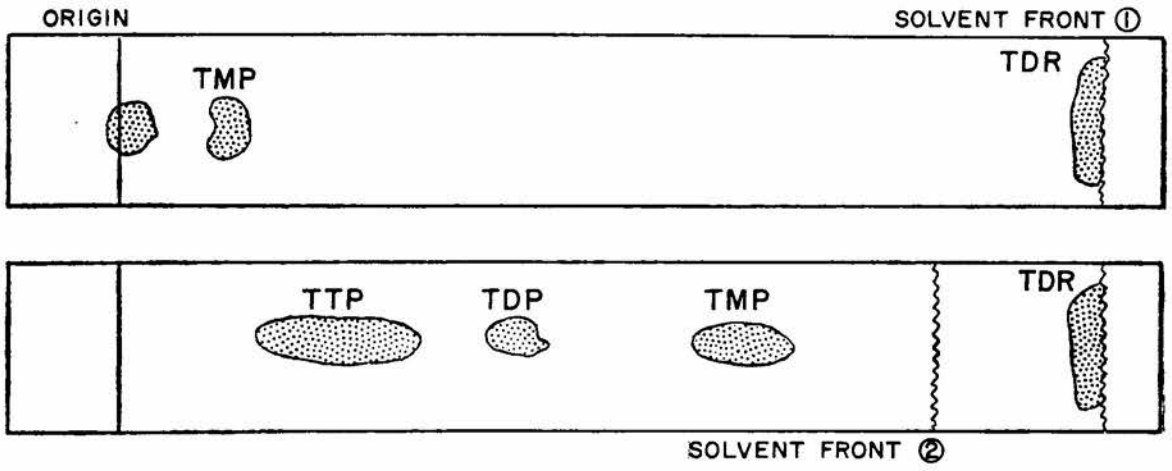


Diagram showing the distribution of the thymidine nucleotides following chromatographic separation using Whatman cellulose paper AE30.

Discussion on the relative merits of the methods used for the separation of thymidine and its phosphorylated derivatives.

The most generally useful method was found to be that of Weissman et al (1960) using cellulose columns. It provided a means of rapid and easy separation of the various fractions and the columns were easily regenerated by washing with water. The fractions could be eluted from the column in a fairly small volume which allowed the radioactivity in 0.2 ml. aliquot to be counted readily by means of a scintillometer.

The use of formate columns with manual elution was tedious and the fractions were contained in somewhat larger volumes. This sometimes necessitated the concentration of the fractions in order to obtain significant numbers of counts above background when the samples were examined for radioactivity. This in turn led to further difficulty because the high concentration of ammonium formate gave rise to self absorption if the counting was done by means of a continuous gas flow counter using stainless steel planchettes and to quenching if the scintillometer was used. A further objection was that the TCA-extracts when applied to the column tended to produce bubbles in the resin and so gave rise to chanelling. This could be overcome by the use of perchloric acid extractions.

When formate columns were used in conjunction with gradient elution and with perchloric acid extracts the results were very satisfactory and the fractions were recovered in reasonably small volumes. It was found generally, however, to be more time consuming than the method employing cellulose columns.

The paper chromatographic method was found to be satisfactory

unless there was a high concentration of radioactive thymidine present, when some trailing over the paper occurred. Moreover, it was again more time consuming than the use of the cellulose columns. The latter method was therefore adopted for the majority of the experiments and proved to be very satisfactory.

Determination of total radioactivity in each nucleotide fraction.

Whereas complete separation of excess ^{3}H-thymidine from the nucleotide fractions could be readily achieved, difficulty was encountered in attaining the removal of excess ^{14}C-formate. Hence, in studies with ^{3}H-thymidine, the total radioactivity in each nucleotide fraction was measured in a windowless flow counter (following the plating of appropriate aliquots in the centre of a stainless steel planchette) or in a Packard liquid scintillometer. In all studies with ^{14}C-formate, because of the known contamination with non-thymine radioactive substances in each of the separated fractions composed of the individual thymine nucleotides, the following procedure was adopted. Subsequent to the addition of thymine (1 µmole) to each fraction, the solution was evaporated to dryness and the residue was digested with 0.2 ml. concentrated perchloric acid for 1 hour in a boiling water bath, in order to hydrolyse the thymine-containing nucleotides to free thymine. After digestion the solution was diluted 10 times and neutralised with KOH. Thymine was separated from other bases in the supernatant and from ^{14}C-formate by paper chromatography in the isopropanol-hydrochloric acid system; this separation was followed by re-chromatography in the

butanol-ammonia system. Following elution of the thymine from the paper its concentration was determined by measuring the extinction in a Beckman ultraviolet spectrophotometer and the radioactivity was measured in the manner described above. This information permitted the calculation of the amount of radioactivity in 1 umole of thymine and hence the total amount in each fraction associated with the thymine-containing nucleotides.

Nucleic Acid Extraction.

(a) ^{3}H-thymidine incubations:

The procedure of Schneider was used. A hot TCA extract using 5-10 cc. of 5 per cent TCA at 90°C for 30 minutes was made on the cells following complete extraction of the low molecular weight compounds by cold TCA. The amount of radioactivity present in an 0.2 ml. aliquot was then determined in a scintillometer or continuous gas flow counter (after removal of the TCA by ether extraction). The specific activity of the DNA-thymine was then determined by estimating the DNA content of the sample (see below).

(b) ^{14}C-formate incubations:

The nucleic acid extract was made as above and then evaporated down to dryness. Hydrolysis with $HClO_4$ was performed, no additional thymine having been added in this instance. DNA-thymine was then separated by paper chromatography and the specific activity determined as outlined above.

Estimation of the DNA content of the Nucleic Acid Extract.

In these experiments the DNA content was measured by colour reactions of the sugar component. A major difficulty of such methods

is the calculation of the actual amount of DNA present from the amount of reactive deoxypentose. Comparative results, however, were all that were required for the experiments to be described. Two methods were employed. The modified method of Dische (1930) described by Burton (1956) and the Stumpf (1947) reaction. The former method was found to be more sensitive but the latter was easier to carry out and was sensitive enough for the relatively large amounts of DNA involved.

(a) Modified Dische Technique.

This method depends upon the development of a blue colour when diphenylamine in a mixture of acetic acid and sulphuric acid reacts with sugar residues which were originally combined with DNA bases (probably purines).

Diphenylamine reagent.

1.5 g. of steam distilled diphenylamine was dissolved in 100 ml. redistilled glacial acetic acid and 1.5 ml. conc. H_2SO_4 added. The reagent was made up fresh before each experiment and immediately before use 0.1 ml. of aqueous acetaldehyde in a concentration of 16 mg. per ml. was added to each 20 ml. of the reagent.

DNA Standard Solution.

40 mg. of a standard preparation of high molecular weight DNA were dissolved in 100 ml. of 0.05 N NaOH. From this a working standard was prepared in the following manner. Equal volumes of the standard DNA solution and perchloric acid were heated for 15 minutes at 70°C to hydrolyse the highly polymerised DNA and a fresh solution

was prepared every 3 weeks.

Procedure.

To 0.96 ml. of the nucleic acid extract from which the trichloracetic acid had been extracted by ether, was added 0.04 ml. concentrated perchloric acid to give a final concentration of 0.5 N. To this was added 2 ml. of the diphenylamine reagent. A blank tube was prepared containing no DNA and 5 standard tubes containing the working standard DNA solution in volumes ranging from 0.2 to 1.0 ml. and 2 ml. diphenylamine reagent were prepared. The final volume was adjusted to 3 ml. with 0.5 N perchloric acid. All tubes were set up in duplicate and incubated at 37°C overnight for 16-20 hours. A blue colouration developed depending upon the concentration of DNA present and the colour intensity was read by measuring the optical density against the blank at 600 mμ. This was compared with the values obtained with the standard DNA solutions.

(b) Stumpf Reaction.

In this method a pink colour develops when DNA is heated with cystcine and sulphuric acid. To a test tube was added 0.05 ml. of 5% cystcine hydrochloride, an aliquot of the unknown solution (0.5 ml.) and 5 cc. 70 per cent H_2SO_4. The mixture was stirred rapidly with a glass rod and after standing at room temperature the optical density was measured at a wave length of 490 mμ. on the Klett-Summerset spectrophotometer. A reagent blank was used to set the instrument scale to zero and the results quantitated by means of a standard curve obtained at the same time under identical conditions. The standard material used was thymidine in amounts varying from 0 to ·25 mg. per

test tube and this was equated with a "unit" of DNA which was the amount giving the same colour reactions as 1 mg. thymidine.

Preparation and incubation of cell free extracts from mouse leukaemia cells.

Cells were harvested 6 days following the innoculation of AKR x DBA/2 F_1 hybrid mice with L5178Y ascites cells. After centrifugation the cells were washed once with normal saline. Packed cells (3 ml.), buffer solution (0.05 M, pH 7.9) and glass beads (200 microns, 7 ml.) were agitated for 20 seconds in a Nossal vibrator. Following filtration through glass wool the filtrate was centrifuged at 10,000 x g. for 10 minutes. Both the composition of the incubation mixture and the conditions used were similar to those described by Weissman et al (1960). The reaction mixture (1.2 ml.) contained $MgCl_2$ (35 µmoles), ATP (110 µmoles), tris buffer pH 7.9, (110 µmoles), ^{3}H-thymidine (5 µc, 0.01 µmole), and various levels of IUdR (0.01, 0.1 and 0.5 µmoles). The reaction was terminated after incubation for 90 minutes at 37°C by the addition of TCA in a final concentration of 5 per cent. The TMP fraction was then separated using cellulose columns as described above.

In Vivo studies to test the inhibitory capacity of IUdR and ICdR on tumour growth.

Female (AKR x DBA/2) F_1 hybrid mice, 6-8 weeks old and weighing 18-25 g. were used. The animals were fed Purina Laboratory Chow, and drinking water was available ad libitum. Transmission of the leukaemia which was maintained in the ascites form was accomplished by a method modified after that of Lowenthal and Jahn (1932). One ml.

of ascites fluid containing L5178Y tumour cells on the seventh day of development was removed aseptically from a freshly sacrificed donor mouse and transferred immediately to a test tube containing 9 ml. of sterile Ringer-Locke's solution. The solution contained approximately $3x10^6$ cells per ml. Each mouse was injected with 1 ml. of this fluid subcutaneously into the right flank. Treatment with the drug (IUdR or ICdR) was begun 24 hours later into individually weighed mice. Various doses in a constant volume of 1 ml. per mouse were injected intraperitoneally once daily for 6 consecutive days. In each experiment 10 mice were used to study the effect of each dose level of the drug while 10 mice which served as controls were given 1 ml. of normal saline per mouse simultaneously. On the day following the final dose each mouse was weighed, sacrificed and its tumour dissected out and weighed. The average tumour weight of each group was compared with that of the controls and the result was recorded in terms of percentage inhibition of tumour growth.

STUDIES ON MICROORGANISMS.

The measurement of the ability of IUdR and ICdR to inhibit the growth of microorganisms was carried out by methods similar to the microbiological assay methods employed for vitamin B_{12} and folic acid.

Into each of 7 test tubes was measured 2.5 ml. of double strength growth medium and to this was added the limiting growth factor in the appropriate quantity to allow half maximum growth (folic acid 0.5 ml., 1.8×10^{-5} µm/ml., thymidine 0.5 ml., .04 µm/ml., thymine 0.5 ml., .04 µm/ml.). The antimetabolite (IUdR or ICdR) was added in the following amounts to successive tubes 0.0; 0.25; 0.5; 1;

2.5; 5 and 10 umoles and the final volume adjusted to 5 ml. with distilled water. A blank tube containing no antimetabolite and a further control tube from which the limiting growth factor was witheld were also prepared. All experiments were performed in duplicate.

The contents of all the tubes (other than the blank) were inoculated with the test organism (Streptococcus faecalis ATCC 8043). The tubes were incubated for 16 hours. The amount of growth depends upon two factors: (1) the limiting growth factor in the medium and (2) the amount of inhibition (or stimulation) induced by the analogue. The turbidity of the contents of the tube is proportional to growth and can be compared with the amount of growth in the tube containing no analogue. The turbidity was measured with a Klett Summerson photoelectric colorimeter using filter number 66 (i.e. with light at 660 mμ.).

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CHAPTER VI.

RESULTS AND DISCUSSION.

SECTION I.

EFFECT OF 5-IODO-2'-DEOXYURIDINE ON THE BIOSYNTHESIS OF THE PHOSPHORYLATED DERIVATIVES OF THYMIDINE AND DNA-THYMINE.

Introduction: IUdR has been reported previously to be an effective competitive antagonist of the utilisation of thymidine for the biosynthesis of the TMP portion of DNA (Prusoff, 1959b. and c; Prusoff, 1960 a. and b; Mathias and Fischer, 1959a. and b). Through a limitation on the use of thymidine derivatives, formed from either orotic acid or formate, the appearance of these precursors in DNA-thymine was inhibited by IUdR (Prusoff 1959c. and 1960a). Thus, both the exogenous and the de novo pathways concerned with the formation of phosphorylated derivatives of thymidine were affected by IUdR (or its derivatives). This analogue inhibited the reproduction of mammalian (Prusoff, 1959b; Mathias and Fischer, 1959a. and b; Jaffe and Prusoff, 1960; Cheong et al, 1960) as well as of bacterial cells (Prusoff, 1959c). Although it has been demonstrated that IUdR can be incorporated extensively into the DNA-polymer (Prusoff, 1959b. and 1960b; Mathias and Fischer, 1959a. and b; Jaffe and Prusoff, 1960; Cheong et al, 1960; Eidinoff, 1959c. d. and e.), it was not established that inhibition of cellular reproduction is causally related to this observed biochemical event. The results shown below indicate that the decreased incorporation of precursors into DNA-thymine

is a result of a primary inhibition of thymidine kinase, TMP kinase, or DNA-polymerase, the specific site of the inhibition being characteristic of the cell type.

Mouse Tumours - Lymphoma L5178Y cells in vitro.

(a) With ^{3}H-thymidine as the precursor. In Table I and the accompanying histogram are shown the results obtained from pre-incubation of L5178Y cells with IUdR for 15 minutes prior to the addition of ^{3}H-thymidine. A seven-fold increase in the specific activity of DNA was observed in the presence or absence of IUdR when the incubation was prolonged from 0.5 hr. to 2.0 hr., although at both intervals of time there was an identical degree of inhibition (78 per cent) by IUdR of the utilisation of ^{3}H-thymidine for the biosynthesis of DNA-thymine. In the presence of the analogue, both at 0.5 hr. and at 2 hr., there was a decrease of about 94 per cent in the amount of radioactivity in the combined nucleotide pools. The decreased incorporation of ^{3}H-thymidine into DNA-thymine appears to be a result of an inhibition which occurred prior to the formation of TMP. Two possible mechanisms of inhibition include a block by IUdR of thymidine kinase or of the mechanism of transport of thymidine into the cell. Support for the former hypothesis is described in Page 82.

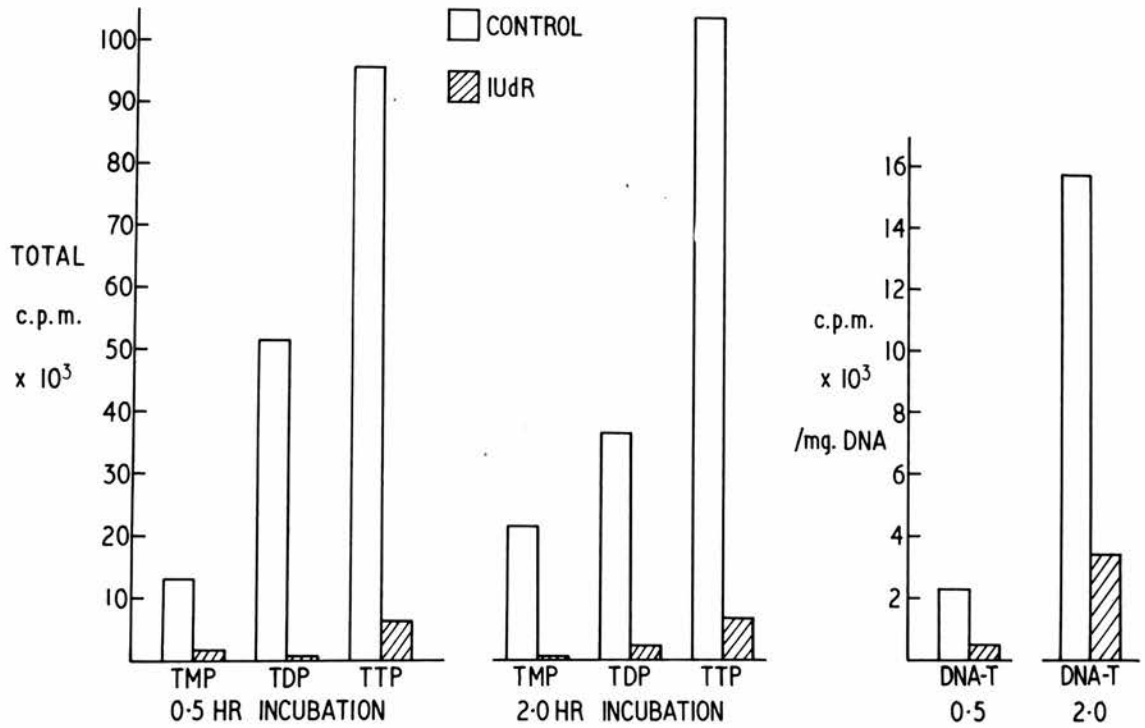
Because thymidine *per se* is not on the pathway for the biosynthesis *de novo* of TMP and since it had been shown previously (Prusoff, 1960a) that IUdR inhibits the appearance of either orotic acid or formate in the thymine of DNA, but not the utilisation of orotic acid for the biosynthesis of DNA-cytosine or of RNA-pyrimidines, it

Table 1 and accompanying Histogram.

The effect of pre-incubation of IUdR on the utilisation of ^{3}H-thymidine for the biosynthesis of TMP, TDP, TTP and DNA-thymine by murine L5178Y leukaemic cells *in vitro* *

Analogue+	Time of incubation. (hr.)	Distribution of radioactivity. TMP (counts/min.)	TDP (counts/min.)	TTP (counts/min.)	DNA-thymine (counts/min. per mg. DNA).
-	0.5	13,000	51,400	95,600	2,280
IUdR	0.5	1,800	830	6,200	490
-	2.0	21,500	36,400	104,000	15,700
IUdR	2.0	650	2,360	6,770	3,400

* Details of incubation conditions are described in the text.
\+ Cells were pre-incubated for 15 min. with IUdR (2 μmoles per ml.) prior to the addition of ^{3}H-thymidine (0.04 μmoles per ml.).



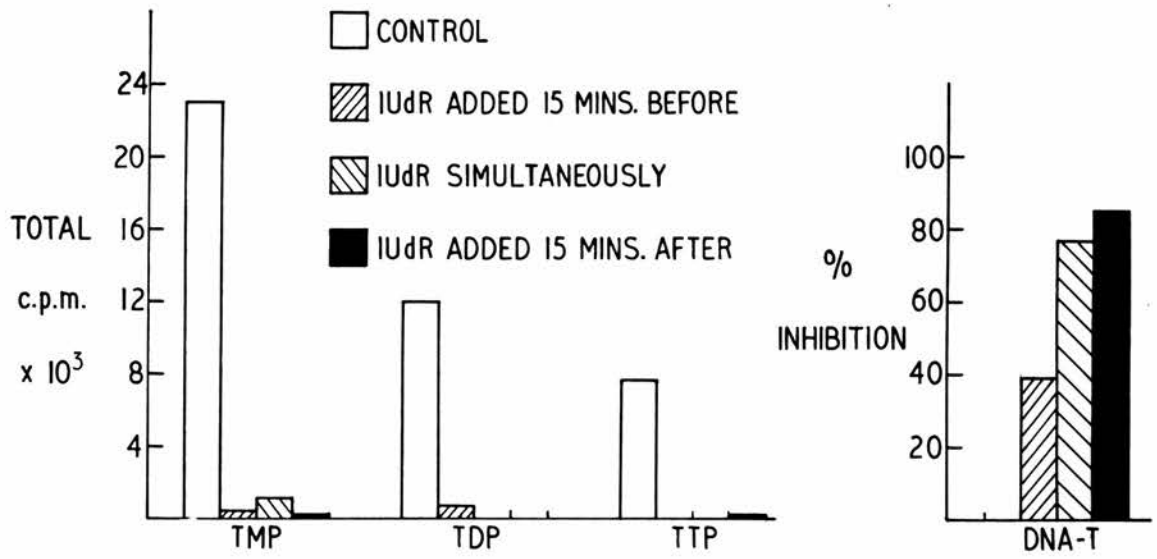
was apparent that IUdR must exert its inhibitory effect at another site, presumably after the formation of TMP.

In a second experiment it was hoped to create conditions whereby the various phosphorylated derivatives of thymidine would have a better opportunity of being formed prior to the development of the inhibitory effect of IUdR. In Table 2 and accompanying histogram are shown the results obtained when IUdR was added to the reaction mixtures before, after or simultaneously with the inclusion of ^{3}H-thymidine; however, there was no significant accumulation of radioactivity in any of the nucleotide pools regardless of the time of addition of IUdR. The variations in the absolute amount of radioactivity in the total acid-soluble pool of the control cells, as well as the different distribution of radioactivity among the nucleotides, as compared with the findings obtained in the previous experiment, may be a reflection of a variation in metabolic activity of different batches of cells. It was found that the extent of inhibition of the incorporation of ^{3}H-thymidine into DNA-thymine varied inversely with the time of addition of IUdR. Thus, the larger incorporation of radioactive thymidine into DNA-thymine was observed when the addition of IUdR followed pre-incubation with ^{3}H-thymidine for 15 minutes. It appeared that the nucleotide fractions were rapidly formed during the 15 minutes interval prior to the addition of IUdR, and that these radioactive pools, formed in the absence of IUdR, were utilised subsequently for the formation of DNA-thymine. Accordingly, the data suggested that the utilisation of ^{3}H-thymidine for the biosynthesis of DNA-thymine by L5178Y cells <u>in vitro</u> is blocked by IUdR primarily at a stage prior to the formation of TMP.

Table 2 and accompanying Histogram.

Effect of time of addition of ^{3}H-thymidine in relation to IUdR on the formation of TMP, TDP, TTP and DNA-thymine by L5178Y cells *in vitro* *

Time of addition of ^{3}H-thymidine in relation to IUdR	Distribution of radioactivity. TMP (counts/min.)	TDP (counts/min.)	TTP (counts/min.)	DNA-thymine (% inhibition)
No IUdR	23,000	12,000	7,700	-
15 min. before	290	700	None	39
Simultaneously	1,200	None	None	77
15 min. after	150	None	110	85



(b) With ^{14}C-formate as the precursor.

For the reasons described above a study was made of the effect of IUdR on the formation of thymine-nucleotides derived from the de novo pathway in which thymidine per se does not participate. In Table 3 and its histogram are shown the effect of IUdR on the utilisation of ^{14}C-formate for the biosynthesis of TMP, TDP, TTP and DNA-thymine by L5178Y cells in vitro.

In agreement with earlier studies (Prusoff, 1960a), marked inhibition of the utilisation of formate for the biosynthesis of DNA-thymine was observed. There was no inhibition in the conversion of TDP to TTP, since in the presence or absence of IUdR there was about a two-fold increase in the amount of radioactivity in the TTP fraction, as compared to that in TDP. Comparison of the TMP and TDP fractions revealed a 100 per cent increase in the amount of radioactivity in the TDP-fraction of the control cells, a 50 per cent increase in the presence of the lower concentration of IUdR, but no increase in the TDP fraction at the higher level of IUdR. In fact, in the presence of the higher concentration of IUdR, the amount of radioactivity of the TDP fraction decreased to 36 per cent of that present in the TMP fraction.

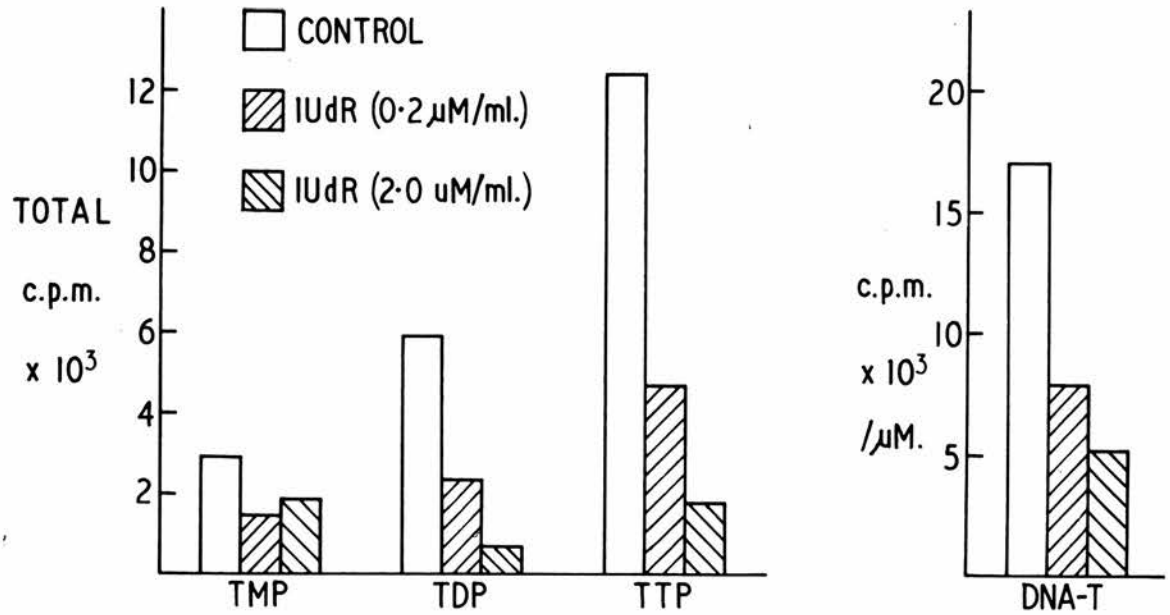
This latter inhibition appears to be a reflection of the decreased formation of TMP. Although a reproducibly larger amount of radioactivity appeared in the TMP fraction in the presence of the higher level of IUdR, this probably reflects an inhibition of TMP kinase with consequent piling up of TMP behind the block. At the lower level of IUdR no inhibition of TMP kinase was observed;

Table 3 and accompanying histogram.

The effect of IUdR on the utilisation of ^{14}C-formate for the biosynthesis of TMP, TDP, TTP and DNA-thymine by L5178Y leukaemic cells *in vitro* *

Concentration of IUdR (μmoles/ml.)	Distribution of radioactivity. TMP (counts/min.)	TDP (counts/min.)	TTP (counts/min.)	DNA-thymine (counts/min per μmole)
None	2900	5900	12,400	17,000
0.2	1480	2360	4700	7,800
2.0	1880	675	1800	5,100

* Details of incubation conditions are described in the text.



hence, the relative distribution of radioactivity among the TMP, TDP and TTP fractions was similar to that of the control. Thus, almost three times as much radioactivity is observed in the TDP and TTP fractions derived from cells incubated with the lower concentration of IUdR, as compared to those derived from the higher level of IUdR. The decreased formation of TMP by L5178Y cells in the presence of IUdR may be related causally to an inhibition of the thymidylate synthetase reaction. In support of this it may be stated that in the case of another thymidine analogue, bromodeoxyuridylic acid, Flaks and Cohen (1959), using a cell free system, observed some degree of inhibition of this reaction although, as would be expected, the effect was extremely small compared with the inhibition caused by fluorodeoxyuridylic acid.

Cell-free extract of L5178Y leukaemia cells:

(a) With ^{3}H-thymidine as the precursor -

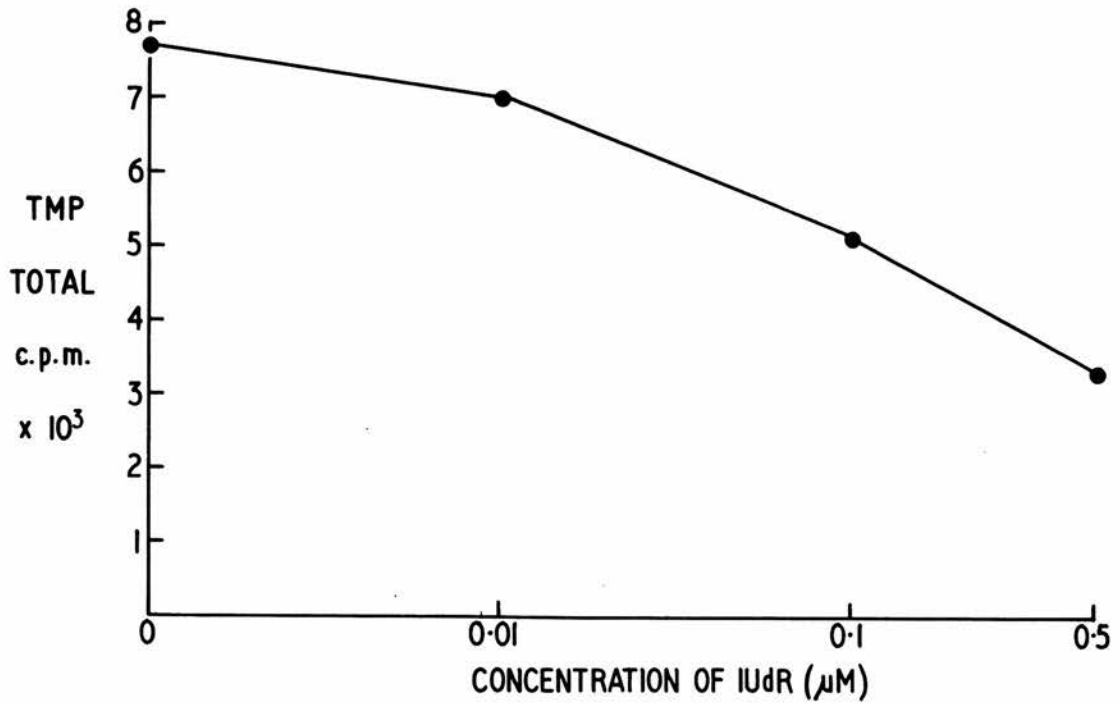
A cell-free extract of mouse L5178Y cells was incubated with ^{3}H-thymidine in the presence of various amounts of IUdR. The results are shown in Table 4 and depicted graphically in Figure 6. At a molar ratio of IUdR to thymidine of 1:1, no decrease in the formation of TMP was observed; at ratios of 10:1 and 50:1, inhibition was observed to the extent of 35 and 57 per cent, respectively. Thus, the decreased utilisation of ^{3}H-thymidine in the presence of IUdR by the corresponding whole cell preparation described above may be explained by the observed inhibition of thymidine kinase. Whether or not there is in addition, any effect on the mechanism of transport of thymidine into cells was not investigated.

Table 4 and Figure 6.

Effect of IUdR on the phosphorylation of ^{3}H-thymidine in a cell-free preparation of murine leukaemia L5178Y cells.

Concentration of IUdR * (µmoles)	TMP. (Total counts/min.)
None	7700
0.01	7000
0.1	5100
0.5	3300

* Amount of IUdR in reaction mixture (1.2 ml.) which contained thymidine (0.01 µmole, 0.5 µC). Details are described in text.



Murine Ehrlich ascites carcinoma cells in vitro:

(a) With ^{3}H-thymidine as the precursor -

In contrast to the observations made with L5178Y cells, IUdR exerted no inhibition in the Ehrlich ascites carcinoma cells, with respect to either the transport of ^{3}H-thymidine into the cells or its subsequent phosphorylation by thymidine kinase (See Table 5 and its accompanying histogram). In addition, there was no inhibition of the conversion of TMP to TDP or of TDP to TTP; rather, there appeared to be an accumulation of cold acid-soluble nucleotides, particularly at the triphosphate level. In agreement with previous studies, there was a marked reduction in the specific activity of DNA-thymine. Thus, the block of the utilisation of thymidine appears to be at the triphosphate level in these cells.

Calf Thymus:

(a) With ^{3}H-thymidine as the precursor -

It was of interest to determine at which site or sites inhibition of the utilisation of ^{3}H-thymidine occurred in normal cells, such as the lymphoid tissue of calf thymus. The results are shown in Table 6 and its histogram. Although there was a 50 per cent decrease in the formation of TMP in the presence of IUdR, a more marked inhibition was observed in the phosphorylation of TMP to TDP. Based on the relative amounts of radioactivity in the TMP and TDP fractions of the control, the amount of radioactivity in the TDP fraction derived from cells incubated with IUdR was only about 30 per cent of that which was obtained when thymidylic kinase was not inhibited. There was no inhibition of the phosphorylation of TDP

Table 5 and accompanying Histogram.

The effect of IUdR on the utilisation of ^{3}H-thymidine for the biosynthesis of TMP, TDP, TTP and DNA-thymine by murine Ehrlich ascites cells in vitro *

Analogue	Distribution of Radioactivity. TMP (counts/min.)	TDP (counts/min.)	TTP (counts/min.)	DNA-thymine (counts/min. per mg. DNA)
None	5500	4500	3300	13,900
IUdR	6500	5000	6800	1,800

* Details of incubation conditions are described in the text.

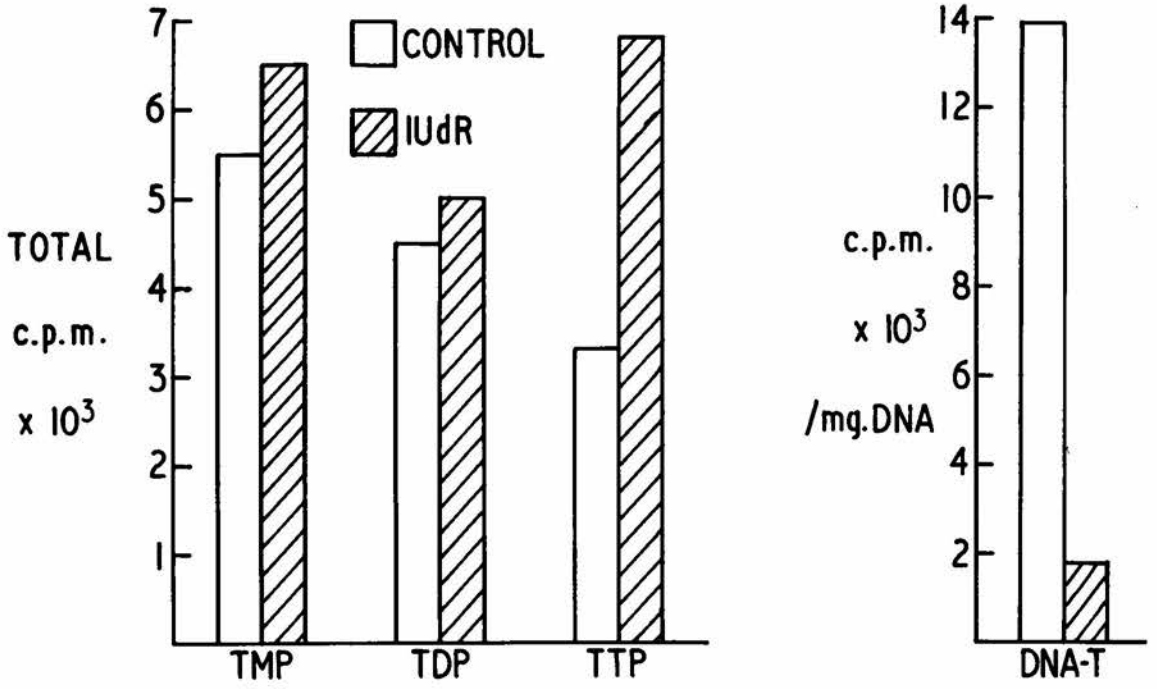
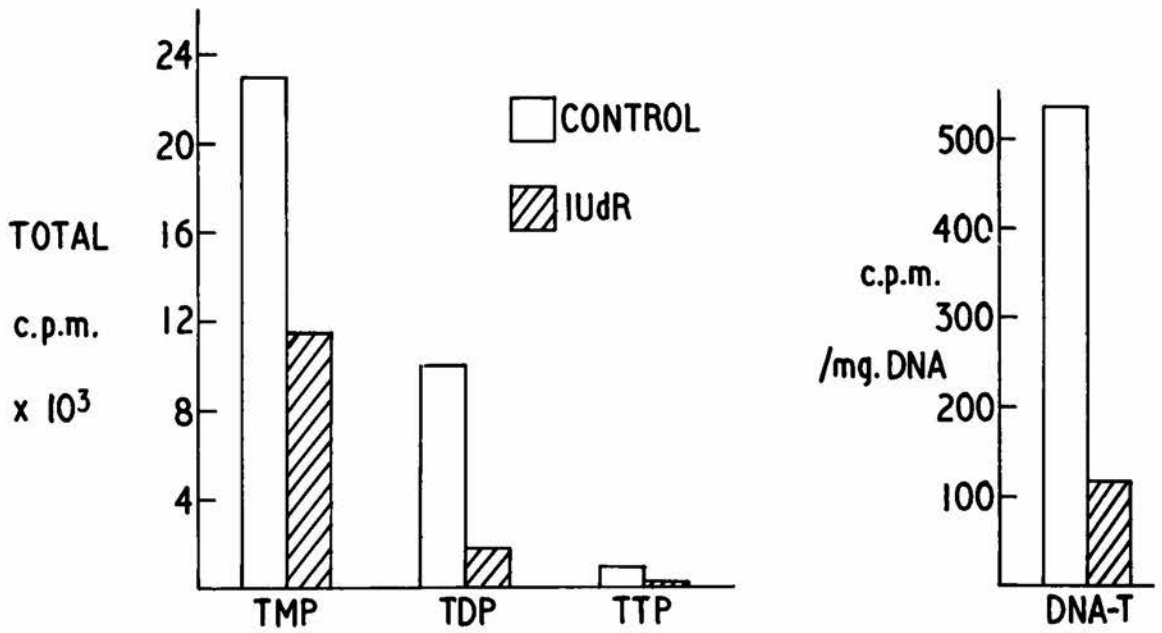


Table 6 and accompanying Histogram.

Effect of IUdR on the utilisation of ^{3}H-thymidine for the biosynthesis of TMP, TDP, TTP and DNA-thymine by normal calf thymus in vitro *

Analogue	Distribution of radioactivity. TMP (counts/min.)	TDP (counts/min.)	TTP (counts/min.	DNA-thymine (counts/min. per mg. DNA)
None	23,000	10,000	980	536
IUdR	11,500	1,800	270	114

* Details of incubation conditions are described in the text.



to TTP or of the utilisation of TTP for DNA-biosynthesis. The 80 per cent decrease observed in the specific activity of DNA-thymine is probably a reflection of the primary inhibition of TMP kinase, coupled with a less marked inhibition of either the uptake of thymidine by the cells or of its initial phosphorylation to TMP.

Human Leukaemia Cells:

The inhibition of the synthesis of DNA-thymine brought about by IUdR in various forms of human leukaemia is shown in Table 7.

(a) Chronic myeloid leukaemia cells - Peripheral leucocytes obtained from two patients with chronic myeloid leukaemia were studied; the results, shown in Tables 8 and 9 and their histograms, indicate that the pattern of inhibition was similar to that seen with murine Ehrlich ascites carcinoma cells. The uptake of ^{3}H-thymidine by the cells was not inhibited by IUdR, nor was its subsequent phosphorylations to TMP, TDP and TTP; however, there was marked interference with the polymerase reaction in which TTP is utilised for the biosynthesis of DNA. Thus, in the presence of IUdR an increase in the amount of radioactivity in the cold acid-soluble nucleotide pools was observed, as well as a marked decrease in the specific activity of DNA-thymine.

(b) Acute monocytic leukaemia cells - The effect of IUdR on the utilisation of ^{14}C-formate and of ^{3}H-thymidine for the biosynthesis of the various thymine-containing nucleotides, as well as its effect on the formation of DNA-thymine, were studied in white blood cells derived from two patients with acute monocytic leukaemia.

The results (see Tables 10, 11 and 12 and their related histograms) indicate that the formation of TMP from ^{3}H-thymidine by

Table 7.

Effect of IUdR on the incorporation of ^{3}H-thymidine into DNA-thymine of various human leukaemias.

Leukaemia.	Percentage inhibition.
Chronic granulocytic	90
" "	54
Plasma cell	80
Acute monocytic	86
" "	88
Chronic lymphocytic	71
" "	79
Leucosarcoma	10

Table 8 and accompanying Histogram.

Effect of IUdR on the utilisation of ^{3}H-thymidine for the biosynthesis of TMP, TDP, TTP and DNA-thymine by human chronic granulocytic leukaemia cells *in vitro* * Patient 1.

Case	Analogue	Distribution of radioactivity. TMP (counts/min.)	TDP (counts/min.)	TTP (counts/min.)	DNA-thymine (counts/min. per mg. DNA.
A.	None	7600	7500	7300	1400
	IUdR	6500	17,500	7200	140

* Details of incubation conditions are described in the text.

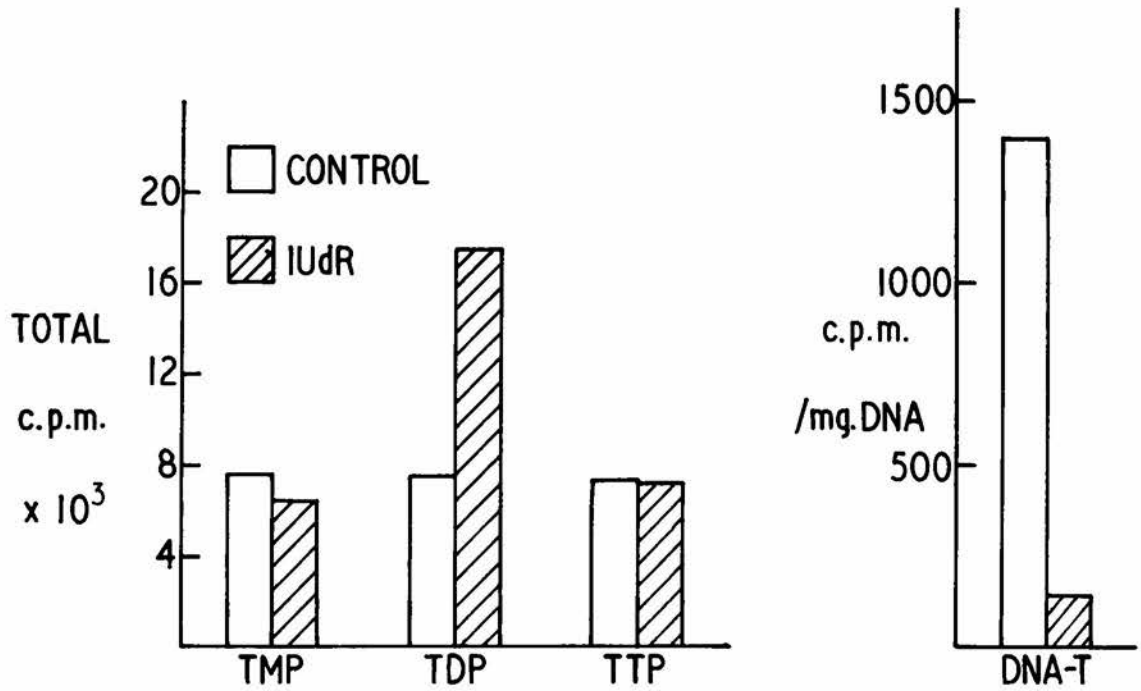


Table 9 ... accompanying histogram.

Effect of IUdR on the utilisation of ^{3}H-thymidine for the ... of ..., ..., TTP and DNA-thymine by human ... blocytic leukaemia cells *in vitro* * ... patient ...

Case	Analogue	Distribution of radioactivity. TMP (counts/min.)	TDP (counts/min.)	TTP (counts/min.)	DNA-thymine (counts/min. per mg. DNA)
B.	None	1400	1300	1100	1400
	IUdR	2700	1400	3200	620

Details of incubation conditions are described in the text.

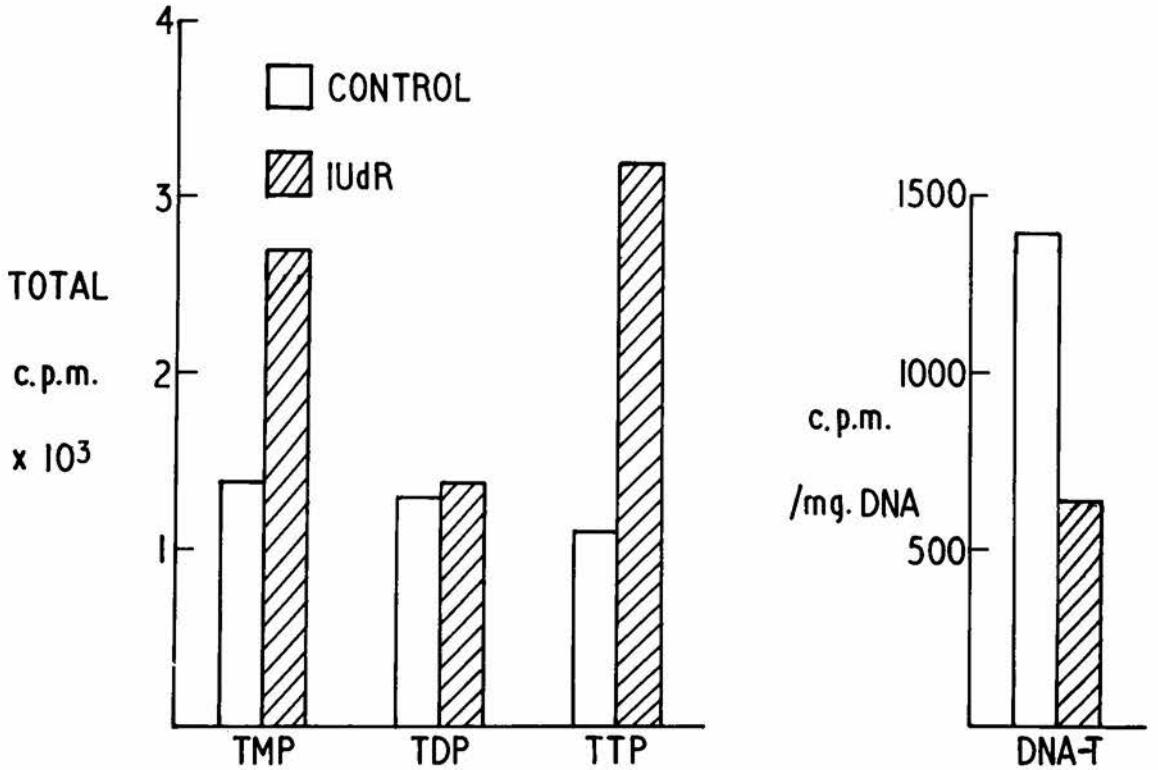


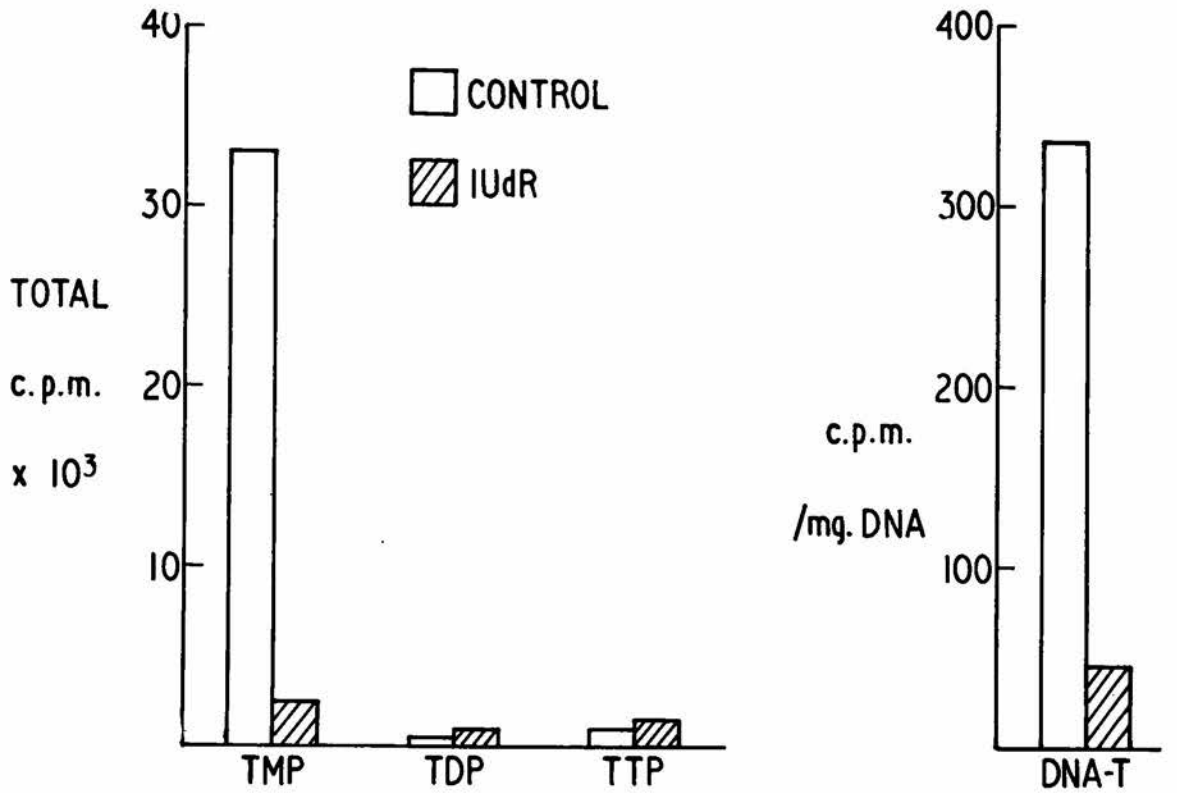
Table 10 and accompanying Histogram.

Effect of IUdR on the utilisation of ^{3}H-thymidine for the biosynthesis of TMP, TDP, TTP and DNA-thymine by human acute monocytic leukaemic cells *in vitro* * - Patient 1.

Analogue	Distribution of radioactivity. TMP^{x} (counts/min.)	TDP^{x} (counts/min.)	TTP^{x} (counts/min.)	DNA-thymine^{+} (counts/min. per mg. DNA)
None	35,000	340	1,100	336
IUdR	2,700	910	1,500	46.

\+ Scintillometer counting

x Gas flow counting.



[illegible] histogram.

Effect of IUdR on the utilisation of ^{3}H-thymidine for the biosynthesis of TMP, TDP, TTP and DNA-thymine by human acute monocytic leukaemic cells in vitro - patient 2.

Analogue	Distribution of radioactivity. TMP^{x} (counts/min.)	TDP^{x} (counts/min.)	TTP^{x} (counts/min.)	DNA-thymine^{+} (counts/min. per unit DNA)^{o}
None	37,000	15,000	1,000	4,700
IUdR	17,000	15,000	800	860

+ Scintillometer counting.

x Gas flow counting.

o cpm in amount of DNA corresponding to 1 mg. of thymidine in Stumpf reaction.

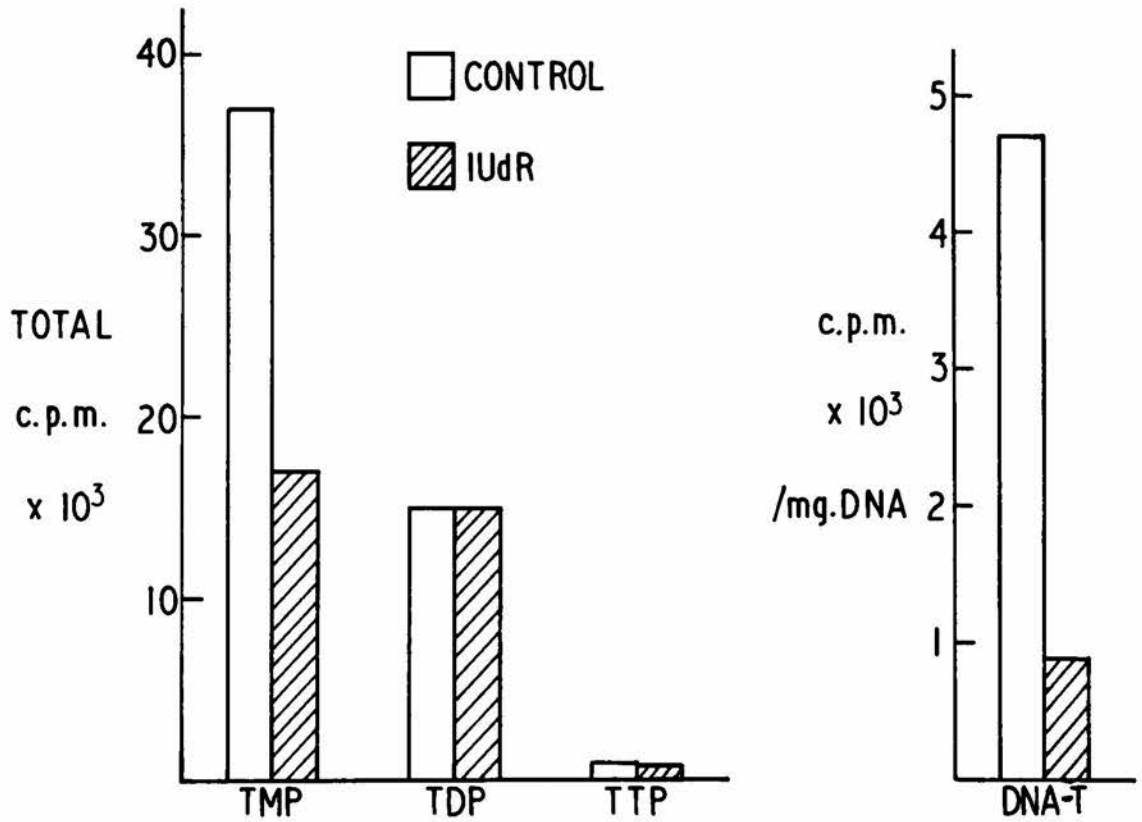


Table 12 and accompanying Histogram.

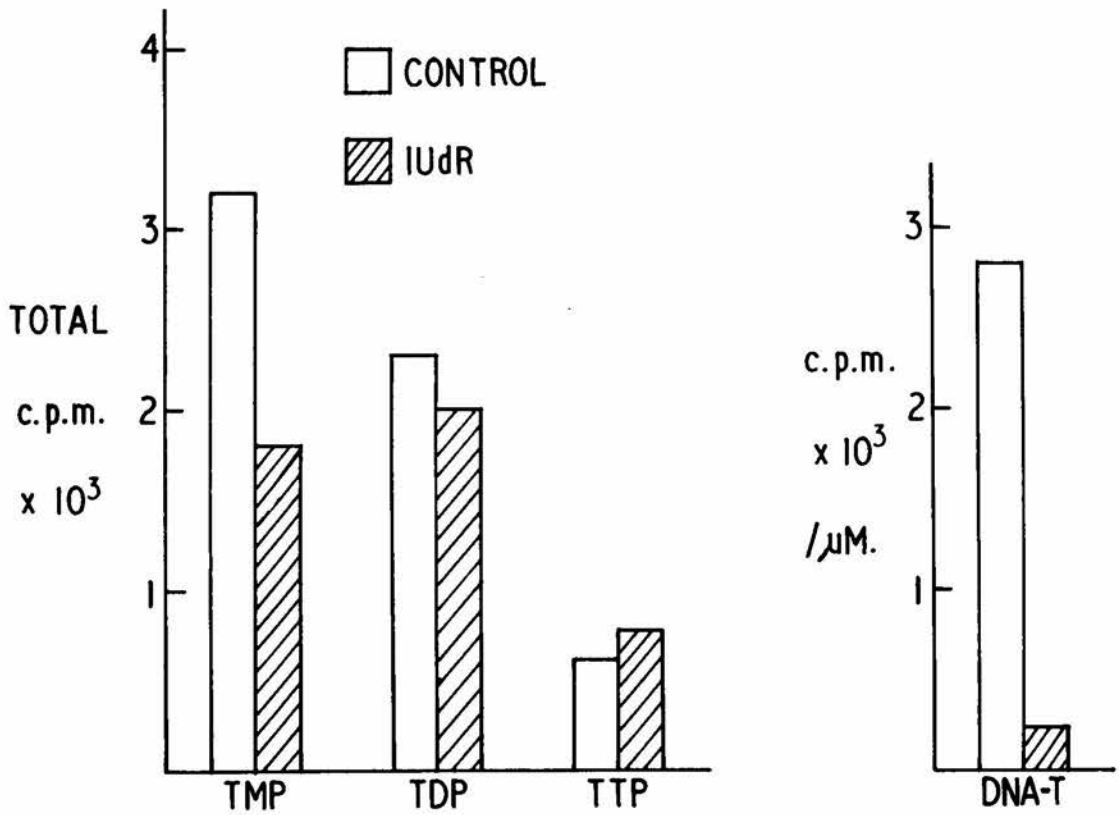
Effect of IUdR on the utilisation of ^{14}C-formate for the biosynthesis of TMP, TDP, TTP and DNA-thymine by human acute monocytic leukaemic cells *in vitro* - Patient 2.

Distribution of radioactivity.

Analogue	TMP[x] (counts/min.)	TDP[x] (counts/min.)	TTP[x] (counts/min.)	DNA-thymine[+] (counts/min. per µm.)
None	3200	2300	620	2800
IUdR	1800	2000	770	230

+ Scintillometer counting.

x Gas flow counting.



the leucocytes of patient 1 was of the same order as that observed with murine L5178Y leukaemic cells; however, in patient 2 the effect on the formation of TMP was much less marked. Although the pools of TMP derived from ^{3}H-thymidine in the absence of IUdR, in patients 1 and 2, were of essentially the same size, there was a marked difference in the amount of radioactivity in the TDP fraction. In comparison with the TMP fraction, the radioactivity in the TTP fractions in either the presence or the absence of IUdR was relatively low. Although there was little difference in the size of the TTP fractions in the presence or absence of IUdR, there was a very marked decrease in the specific activities of the DNA-thymine.

Accordingly, several sites of inhibition by IUdR and its phosphorylated derivatives may be implicated in these cells; (1) a partial block in the formation of TMP derived from the de novo pathway; (2) a partial block in either the transport of thymidine into the cell or of thymidine kinase (the exogenous pathway); and (3) inhibition of DNA polymerase. The last site would appear to be the most critical one in these cells with respect to the inhibition of the biosynthesis of DNA.

(c) Cells of other types of human leukaemia: The effect of IUdR on the utilisation of ^{3}H-thymidine for the formation of TMP, TDP, TTP and DNA-thymine, was examined in cells derived from one case each of chronic lymphocytic leukaemia, and plasma cell leukaemia. In the presence of the analogue a very marked inhibition in the conversion of thymidine to TMP was observed with each type of cell, a finding which might be attributed either to interference with the transport

mechanism or to inhibition of thymidine kinase. Very little radioactivity appeared in TDP, TTP or DNA, even in the absence of IUdR; nevertheless, the amount of ^{3}H-thymidine which appeared in DNA in the presence of IUdR was markedly reduced.

Mechanism of "apparent" resistance to IUdR in vivo.

(a) Walker carcinosarcoma.

In a previous study of the effect of IUdR on the growth of various neoplasms in experimental animals, the Walker carcinosarcoma 256 was reported not to be affected by this analogue (Jaffe and Prusoff, 1960). Accordingly, it was of interest to determine whether the failure to inhibit this tumour is attributable to an inherent resistance to the inhibitory effects of IUdR. An homogenate of this tumour was prepared and the effect of IUdR on the utilisation of ^{14}C-formate and of ^{3}H-thymidine for the biosynthesis of DNA-thymine was investigated. The results (shown in Table 13 and its histograms) indicate that the biosynthesis of DNA is markedly inhibited by IUdR. It is pertinent that neither during nor subsequent to treatment with IUdR were manifestations of toxicity observed in the rats bearing the Walker carcinosarcoma 256. Thus, the apparent resistance of this neoplasm to suppression by IUdR in vivo probably is attributable to an inadequate dosage regimen in this animal species.

(b) Acute monocytic leukaemia.

In a patient with acute monocytic leukaemia, no beneficial effect was noted after a 36 hr. intravenous infusion of IUdR, during

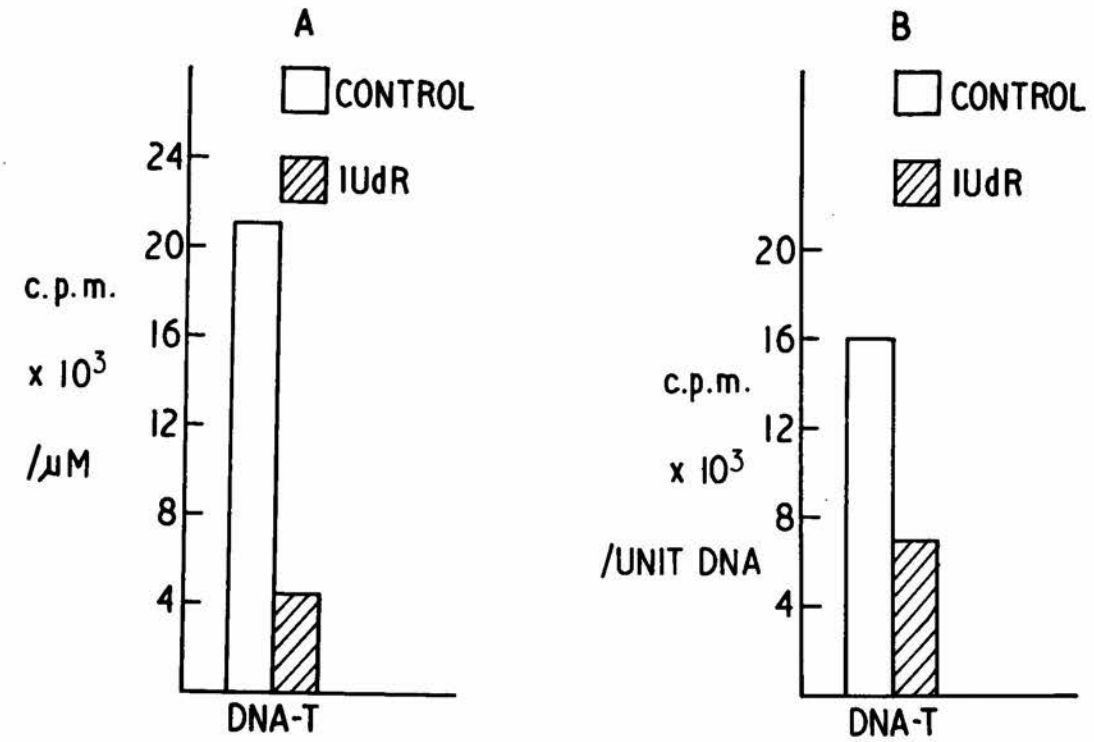
Table 13 and accompanying Histograms.

The effect of IUdR on the utilisation of ^{3}H-thymidine or ^{14}C-formate for the biosynthesis of DNA-thymine by Walker carcinoma 256 cells *in vitro* *

Experiment.	Analogue	Precursor	DNA-thymine (counts/min. per um.)	DNA-thymine. (counts/min. per unit DNA) x
A.	None	^{14}C-formate	21,000	
	IUdR	^{14}C-formate	4,300	
B.	None	^{3}H-thymidine		16,000
	IUdR	^{3}H-thymidine		7,000

* Details of incubation conditions are described in the text.

x Cpm in amount of DNA corresponding to 1 mg. of thymidine in Stumpf reaction.



which time the subject was given a total dose of 300 mg. of IUdR per kg. of body weight. After continuous intravenous infusion of IUdR for 16 hr. samples of the white blood cells of this patient were examined in vitro with respect to their ability to utilise ^{3}H-thymidine for the biosynthesis of DNA-thymine in the presence of various levels of IUdR; the results are shown in Table 14 and its histogram). It is apparent that the extracellular concentrations of IUdR in this patient were inadequate to exert any significant inhibitory effect on the uptake of ^{3}H-thymidine since in vitro the addition of increasing amounts of IUdR resulted in marked inhibition of the biosynthesis of DNA-thymine. It may be concluded, at least tentatively, that if an adequate regimen of dosage with IUdR could be given, which would permit inhibitory concentrations to be attained under in vivo conditions, inhibition of leukaemic cell proliferation should be possible. However, such levels of dosage with IUdR probably could not be administered without causing intolerable effects on normal cells, particularly those of the bone marrow. Of pertinence, however, are studies by Calabresi (1961) which have demonstrated the feasibility of regional protection with arterially infused thymidine, given in small amounts (4 to 8 mg./kg.), while IUdR is administered (110-120 mg/kg.) by intravenous infusion.

Comparison of kinase activities in various mammalian tissues.

During the course of this study, the kinase activities of various tissues have been studied. Although no unequivocal conclusion may be drawn concerning the question as to whether or not

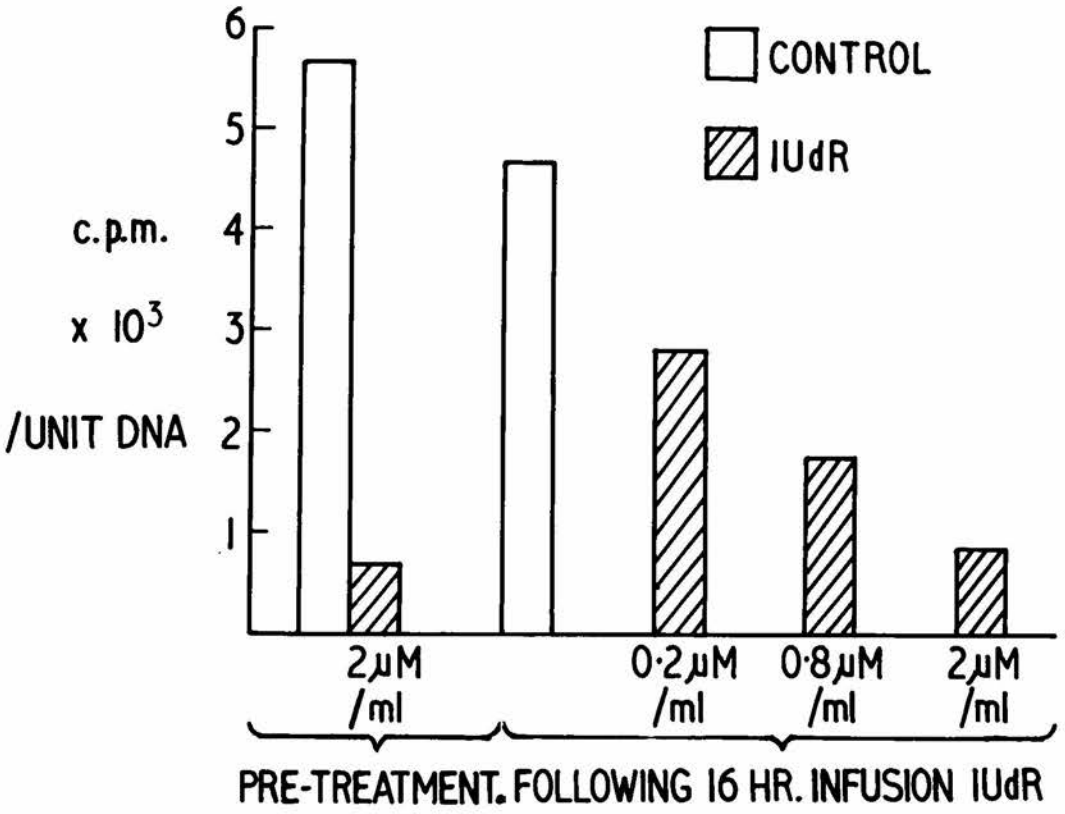
Table 14 and accompanying Histogram.

Comparison of the viability of white blood cells derived from a patient with acute monocytic leukaemia before and during treatment with IUdR to utilise ^{3}H-thymidine for the biosynthesis of DNA-thymine in the presence and absence of IUdR. *

Concentration of IUdR (μmole/ml.)	DNA-thymine. Before treatment (counts/min. per unit DNA)	During treatment (counts/min. per unit DNA) °
None	5600	4650
0.2	--	2800
0.8	--	1800
2.0	680	860

* Details of incubation conditions are described in the text.

° 1 DNA unit is that amount giving same coloured reaction as 1 mg. thymidine in the Stumpf reaction.



TDP is an intermediate in the conversion of TMP to TTP (Weissman et al, 1960b; Bianchi et al, 1961), it is of interest that IUdR had no influence on the ratio of TDP to TTP. If TDP is an intermediate in the formation of TTP, then the corresponding phosphorylated derivative of IUdR, iododeoxyuridine diphosphate, exerts no inhibitory effect on TDP-kinase. Incubation of acute monocytic leukaemia cells (Tables 11 and 12 and related histograms) with radioactive thymidine or formate resulted in the formation of TDP and TTP in the ratio of 15:1 and 4:1 respectively. This suggests that if TDP were not on the direct pathway for the biosynthesis of TTP, then the latter compound is uniquely unstable in this cell population. Bianchi claimed that human tissue contains only small amounts of the enzyme which phosphorylate thymidine to TMP and that all human tissues convert TMP completely into TTP in less than 30 mins. The results shown in Tables 10, 11 and 12 do not support these conclusions. For example, the TMP derived from human acute monocytic leukaemia cells which were incubated for 2 hr. with ^{3}H-thymidine represented in two patients 60 and 94 per cent respectively the radioactivity of the combined TMP, TDP and TTP fractions, whereas the TMP fraction derived from ^{14}C-formate was 50 per cent of the combined fractions. A possible explanation for the differences observed may be that Bianchi derived his conclusions from studies of particle-free fractions, whereas the present studies utilised intact cells.

There are many factors which affect the rate of utilisation of thymidine for the biosynthesis of DNA. Bond et al, 1959 observed extensive labelling of cells from a patient with chronic myeloid

leukaemia when they were incubated *in vitro* with ^{3}H-thymidine, but in chronic lymphocytic leukaemia and myeloma, less than 3 per cent of the cells took up the ^{3}H-thymidine. These authors suggested that the cells with low uptake either have a low turnover rate or lack the ability to utilise thymidine or have an unusually long DNA-synthesising time. Moreover, Craddock (1960) found that the uptake of ^{3}H-thymidine did not correspond with the degree of morphologic immaturity of the cell population. Thus, in myeloproliferative disorders the highest degree of labelling was observed in patients with a low percentage of myeloblasts, while lymphocytes from both acute and chronic lymphatic leukaemia patients showed a low uptake of radioactivity, in contrast to the high degree of uptake by normal thoracic duct lymphocytes. In addition, the uptake was low in two cases of plasma cell in which a large number of blast cells were present. In acute myeloblastic leukaemia it was found that 12 per cent of the cells in the peripheral blood were moderately labelled, a circumstance which indicated that although many cells were synthesising DNA, the rate per cell was quite slow. That too close a correlation should not be drawn between the distribution of radio-activity in the three nucleotide fractions and the rate of DNA synthesis can be seen from later experiments (p.116-123) since the percentage of total radioactivity present in each fraction varies depending upon the length of incubation.

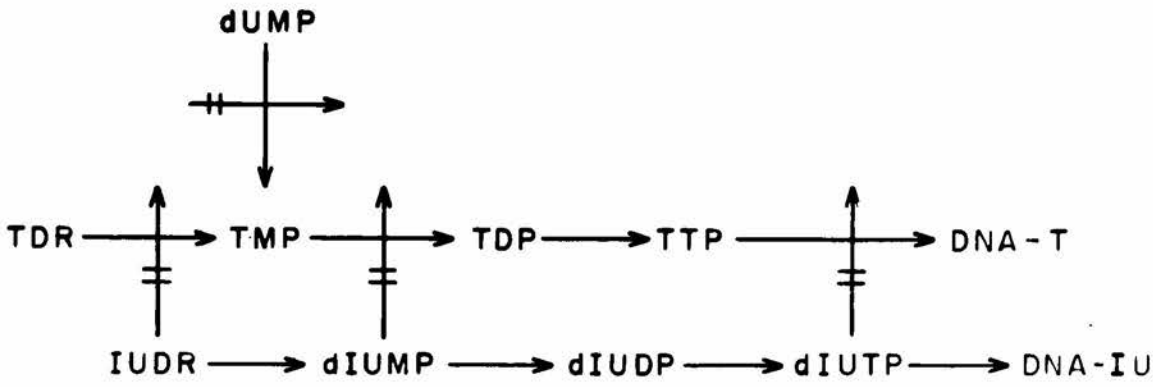
Mechanism of action of 5-iodo-2'-deoxyuridine.

Thymidine monophosphate is the initial compound common to (a) the *de novo* pathway for the biosynthesis of the thymine-component of

DNA, via 2'-deoxyuridylic acid and (b) the exogenous pathway, which is concerned with the utilisation of thymidine derived either from dietary sources, or from the degradation of DNA, or from biosynthetic reactions of other cells or tissues. The appropriate kinases subsequently catalyses the phosphorylation of TMP to TDP and TTP, prior to polymerisation into DNA (Bessman et al, 1958a; Bollum and Potter, 1958b. and 1959; Canellakis and Mantsovanos, 1958; Lehman et al, 1958; Canellakis et al, 1959). It may be inferred that IUdR is converted to the corresponding mono-, di and tri- phosphates, since IUdR has been shown to be incorporated into DNA (Prusoff, 1959b and 1960b, Mathias and Fischer, 1959a; Mathias et al, 1959b; Jaffe and Prusoff, 1960; Cheong et al, 1960; Eidinoff et al, 1959c, d, and e) by replacement of TMP. Inhibition of a specific kinase probably is mediated by the appropriate phosphorylated analogue, e.g. TMP by 5-iodo-2'-deoxyuridine 5'-monophosphate (IUdR-5'-P). This relationship is depicted in Figure 7. Thus, the utilisation of thymidine or of formate for the biosynthesis of DNA-thymine by mouse L5178Y leukaemia cells is blocked by the appropriate derivative of IUdR primarily at the thymidine kinase (reaction 1) and thymidylic acid kinase (reaction 2) respectively; whereas the utilisation of these precursors for the biosynthesis of DNA-thymine in mouse Ehrlich ascites cells is blocked at a single site, DNA-polymerase (reaction 3). It is of interest that inhibition of the conversion of TDP to TTP was not observed in any of the tissues investigated.

An unexpected observation was the decreased formation of thymidylic acid via the <u>de novo</u> pathway suggesting the possibility of

Figure 7.



Metabolic interrelationship of IUDR and precursors of DNA-thymine

the inhibition of the thymidylate synthetase reaction.

The inhibitory effect of IUdR (or more probably its phosphorylated derivatives) is therefore exerted at specific metabolic reaction sites, the primary site being related to the nature of the precursor as well as the species of cell.

SUMMARY.

A study has been made of the effect of IUdR on the formation and utilisation of phosphorylated derivatives of thymidine, as well as on the formation of DNA-thymine, in various murine and human neoplastic

tissues. The decreased incorporation, in the presence of IUdR, of the incorporation of ^{14}C-formate and ^{3}H-thymidine into DNA-thymine is a reflection of an inhibition of the utilisation of thymidine, thymidylic acid or thymidine triphosphate, presumably by IUdR or the corresponding phosphorylated derivatives of IUdR. It is to be noted that the specific metabolic site primarily affected in the various tissues studied is a characteristic of the individual tissue. Thus, DNA-polymerase was primarily inhibited in murine Ehrlich ascites carcinoma and in human chronic granulocytic and acute monocytic leukaemias, whereas studies with murine L5178Y leukaemia cells, using ^{3}H-thymidine and ^{14}C-formate showed a primary blockade of thymidine kinase and thymidylic acid kinase, respectively. Confirmation of the inhibition of thymidine kinase was obtained with a cell-free extract of the L5178Y cells. With normal calf thymus, thymidylic acid kinase, but not thymidine kinase, was inhibited primarily. The apparent resistance to IUdR of the Walker carcinosarcoma 256 of the rat, <u>in vivo</u>, as well as of a patient with acute monocytic leukaemia, could be explained by an inadequate dosage regimen, since marked inhibition of the biosynthesis of DNA by these tissues could be demonstrated <u>in vitro</u>.

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SECTION II.

COMPARATIVE STUDIES WITH 5-IODO-2'-DEOXYURIDINE AND 5-IODO-2'-DEOXYCYTIDINE.

Introduction:

With the advent of ICdR it was important to compare its effectiveness with IUdR as a thymidine antagonist both by *in vitro* and *in vivo* studies and to determine whether or not it offered significant advantages over the uracil derivative. It was hoped that ICdR might show greater metabolic stability (Verdier and Potter, 1960) thus enabling a greater concentration of the drug to be incorporated into the DNA of proliferating cells. Moreover, the presence of the enzyme deoxycytidylate deaminase in certain neoplastic tissues and its absence from most normal tissues offered the possibility of the preferential utilisation of ICdR by malignant cells. Unfortunately, the results shown below do not substantiate these hopes and suggest that certain normal tissues do deaminate ICdR and that certain neoplastic cells do not. For example, the following results show that ICdR is much less effective than IUdR as an inhibitor of the synthesis of DNA-thymine by mouse lymphoma L5178Y cells *in vitro* but *in vivo* it is only slightly less effective as an inhibitor of tumour growth suggesting that in this instance the prior deamination by some other tissue or organ (such as the liver or kidney) is essential before maximum therapeutic efficiency is obtained.

Effect of IUdR and ICdR on the growth of Streptococcus faecalis (ATCC 8043).

Organisms were grown in a medium in which the limiting factor

was thymine, thymidine or folic acid, this metabolite being present in sufficient concentration to allow half maximum growth in each instance. The effect of the analogue to compete with these metabolites was tested by the addition of the analogue in increasing concentrations to the medium. The results are shown in Figures 8, 9 and 10 respectively.

Figure 8.

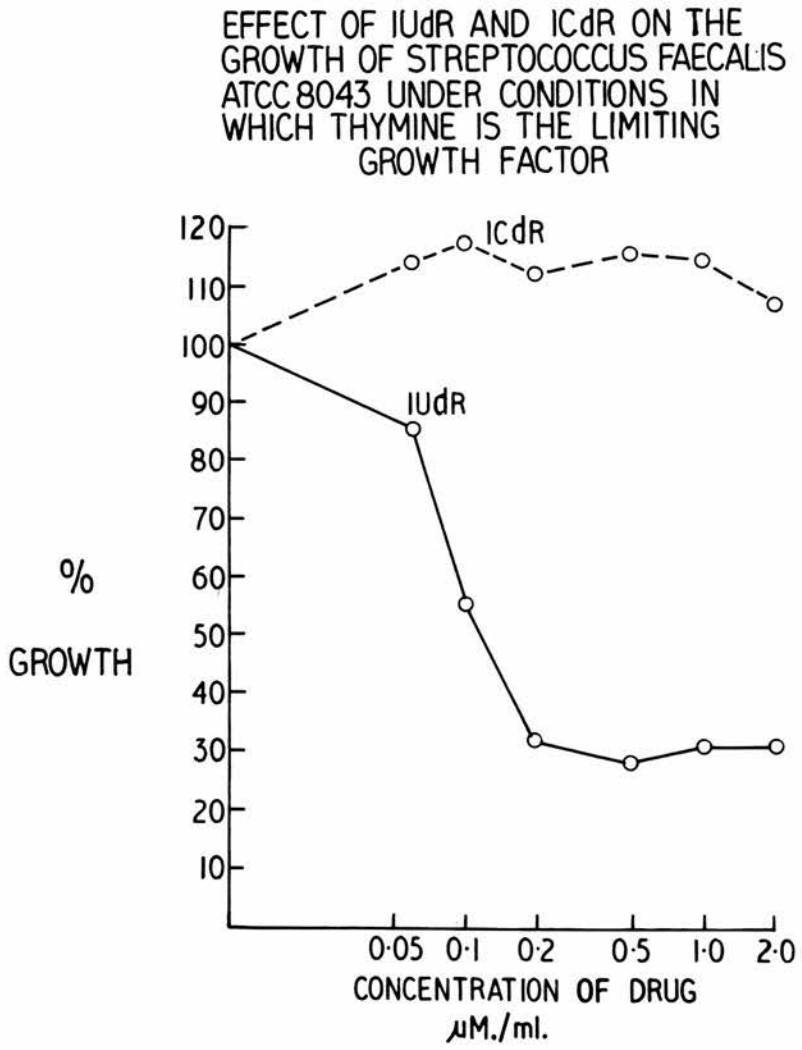


Figure 9.

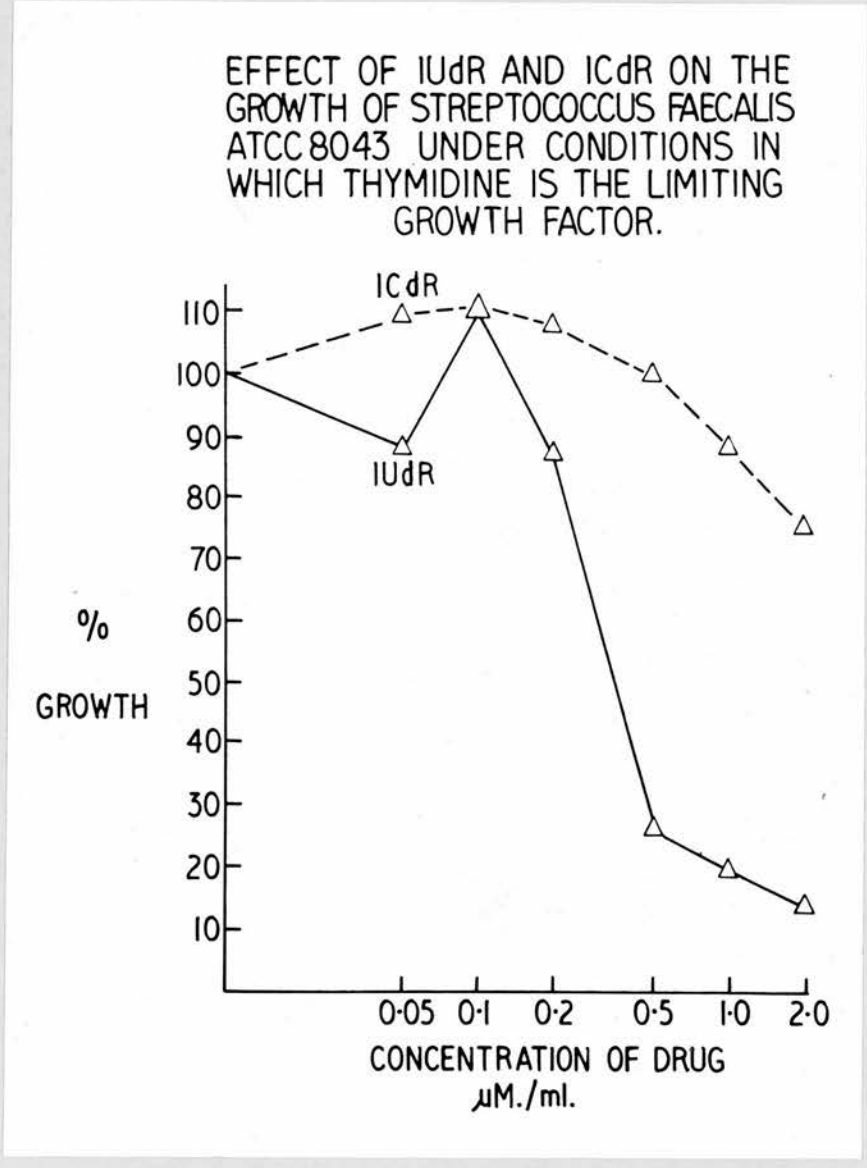
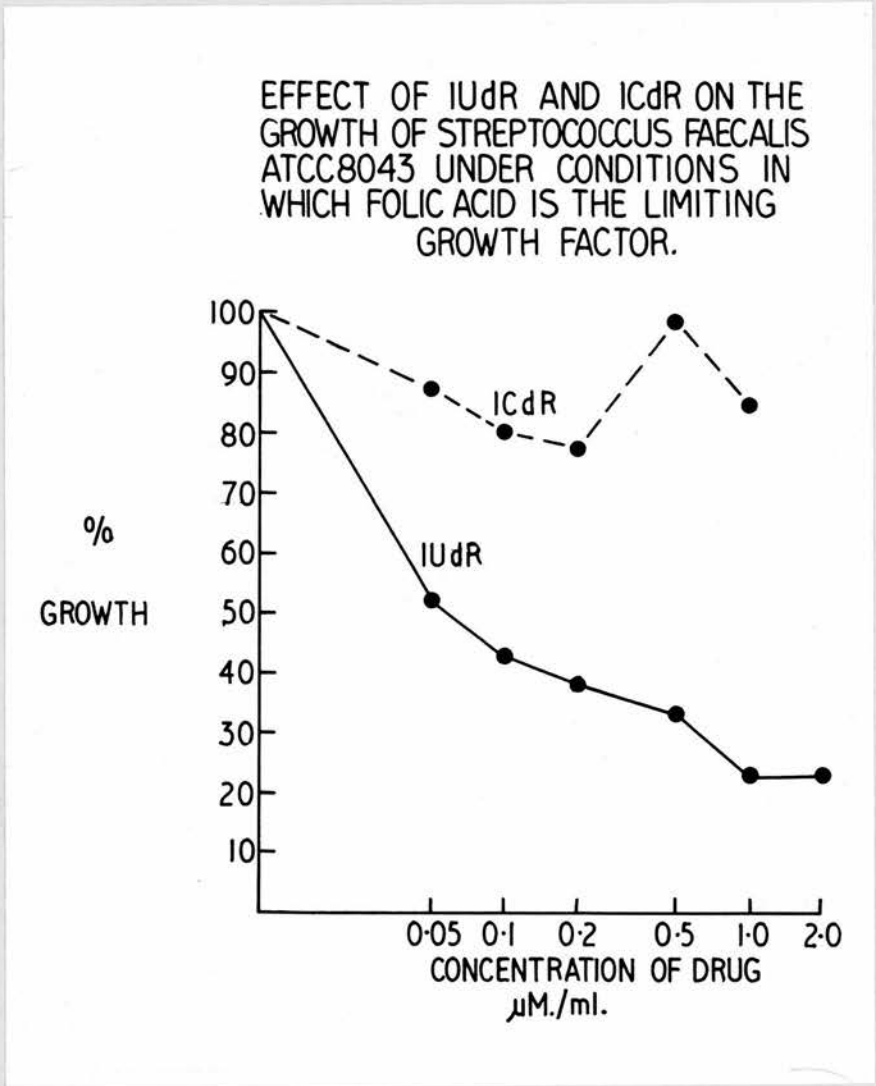

EFFECT OF IUdR AND ICdR ON THE GROWTH OF STREPTOCOCCUS FAECALIS ATCC 8043 UNDER CONDITIONS IN WHICH THYMIDINE IS THE LIMITING GROWTH FACTOR.
ICdR
IUdR
%
GROWTH
110
100
90
80
70
60
50
40
30
20
10
0·05
0·1
0·2
0·5
1·0
2·0
CONCENTRATION OF DRUG
µM./ml.

Figure 10.


EFFECT OF IUdR AND ICdR ON THE GROWTH OF STREPTOCOCCUS FAECALIS ATCC8043 UNDER CONDITIONS IN WHICH FOLIC ACID IS THE LIMITING GROWTH FACTOR.
%
GROWTH
100
90
80
70
60
50
40
30
20
10
ICdR
IUdR
0·05 0·1 0·2 0·5 1·0 2·0
CONCENTRATION OF DRUG
µM./ml.

It will be readily appreciated that while IUdR caused increasing inhibition with increasing concentration, ICdR did not. There was, however, a reproducible slight inhibition of growth when ICdR was added in a concentration of 2 μmoles/ml. to the medium in which thymidine was the limiting growth factor.

The reason for the failure of ICdR to inhibit growth was not investigated but the most likely explanation is that the organisms were unable to deaminate the analogue to the corresponding uracil derivatives. Other possibilities such as failure of transport into the cell cannot be excluded.

Effect of IUdR and ICdR on the growth of the solid tumour form of mouse lymphoma L5178Y in vivo.

Experiments were performed in mice at two different dosage schedules for each drug. The capacity of each drug to inhibit the growth of the tumour was estimated using the method described on p.72. The results are shown in Table 15. From these results it would appear that ICdR is slightly less inhibitory than IUdR, the percentage tumour inhibition at the 9 μm. dose level being 60 per cent and 78 per cent respectively. There was also less toxicity than with IUdR but it has previously been shown that IUdR may be used in this dosage without giving rise to any mortality (Jaffe and Prusoff, 1960). It has since been reported, however, that in acute and chronic toxicity studies in mice, ICdR is less toxic than IUdR (Cramer et al, 1962).

Effect of IUdR and ICdR on the biosynthesis of the phosphorylated derivatives of thymine and DNA-thymine.

(a) Ehrlich Ascites Carcinoma Cells in vitro.

Table 15.

The effect of IUdR and ICdR on the growth of the solid tumour form mouse lymphoma L5178Y in vivo.

	Drug Dosage Level µm/mouse/day	Average Change Body weight	Mortality	Average Tumour Weight	Approx. % Inhibition.
IUdR	9	- 2.0	4/10	50	78
	3	+ 0.8	2/10	134	41
ICdR	18	- 2.4	0/10	31	97
	9	- 1.6	0/10	91	60
Control	-	+ 0.6	0/10	226	-

1. With ^{3}H-thymidine as the precursor:

The results shown in Table 16 and its accompanying histogram indicate that ICdR is less effective than IUdR as an inhibitor of the biosynthesis of DNA-thymine; the inhibition produced by ICdR being only 55 per cent while that due to IUdR was 87 per cent. Neither drug appeared to inhibit the biosynthesis of the nucleotide fractions suggesting that in both cases the primary site of inhibition was at the polymerase stage. It is of interest, however, that the accumulation of radioactivity in the nucleotide pools behind the site of metabolic inhibition was considerably greater in the case of ICdR than IUdR. The total radioactivity in the nucleotide pools was increased by 40 per cent over that in the control cells in the case of IUdR and 80 per cent in the case of ICdR.

Effect of IUdR and ICdR on the biosynthesis of DNA-thymine.

(a) Mouse lymphoma L5178Y cells in vitro.

1. With ^{3}H-thymidine as the precursor -

In Table 17 and the associated histogram are shown the results obtained from a 2 hours incubation of lymphoma L5178Y cells in vitro with IUdR and ICdR. It can be readily seen that ICdR under these conditions is much less effective than IUdR as an inhibitor of DNA-thymine, the percentage inhibition being approximately 38 and 97 respectively.

When ^{14}C-formate was used as the precursor the results were very similar to those with thymidine as the precursor (Table 18 and its histogram). In a further experiment in which the cells were

Table 16 and accompanying Histogram.

Effect of IUdR and ICdR on the utilisation of ^{3}H-thymidine for the biosynthesis of TMP, TDP, TTP and DNA-thymine by Ehrlich ascites cells *in vitro*.

Analogue	TMP	TDP	TTP	DNA-thymine cpm/unit DNA [o]
None	5500	4500	3300	55,500
IUdR	6500	5250	6750	7200
ICdR	8000	9250	8750	25,200

[o] cpm in amount of DNA corresponding to 1 mg. thymidine in the Stumpf reaction.

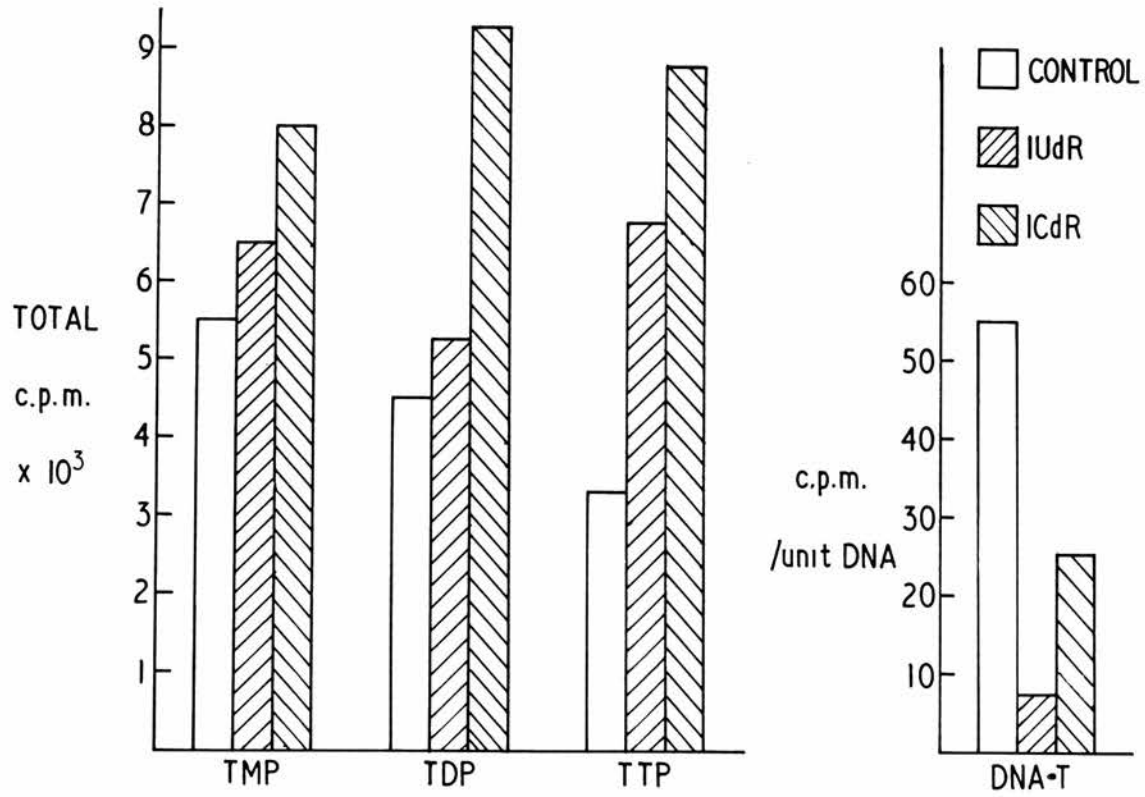


Table 17 and accompanying Histogram.

Effect of IUdR and ICdR on the utilisation of ^{3}H-thymidine for the biosynthesis of DNA-thymine by mouse lymphoma L5178Y cells in vitro.

Analogue	DNA-thymine cpm/unit ^{o} DNA.
None	218,000
IUdR	6,900
ICdR	200,000

^{o} cpm in amount of DNA corresponding to 1 mg. thymidine in the Stumpf reaction.

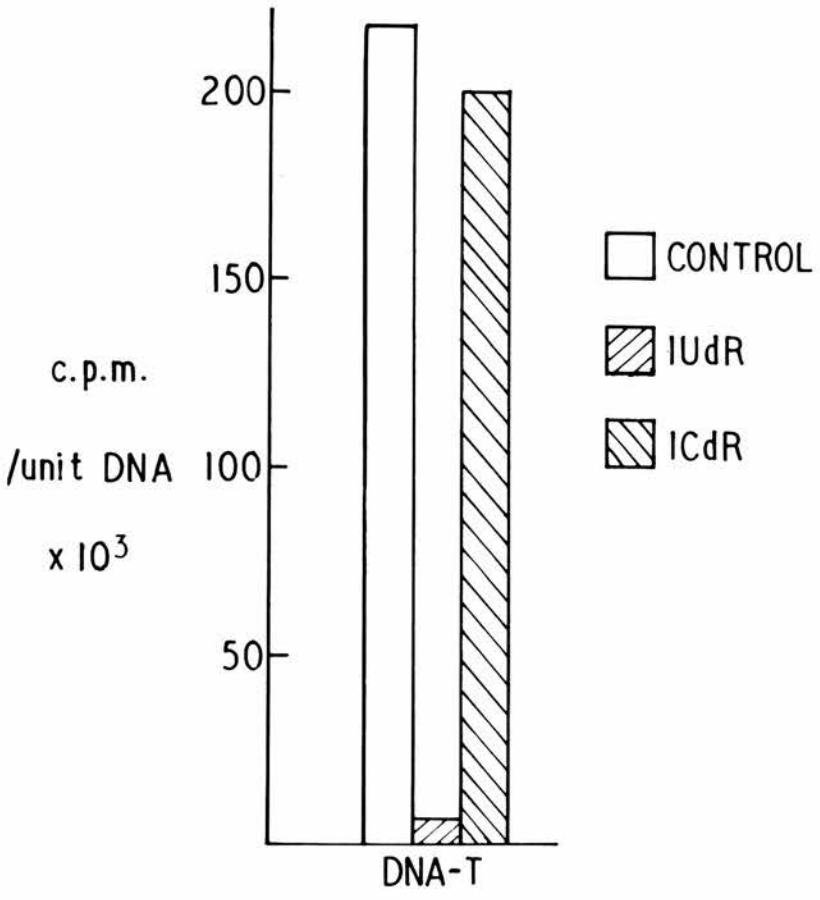
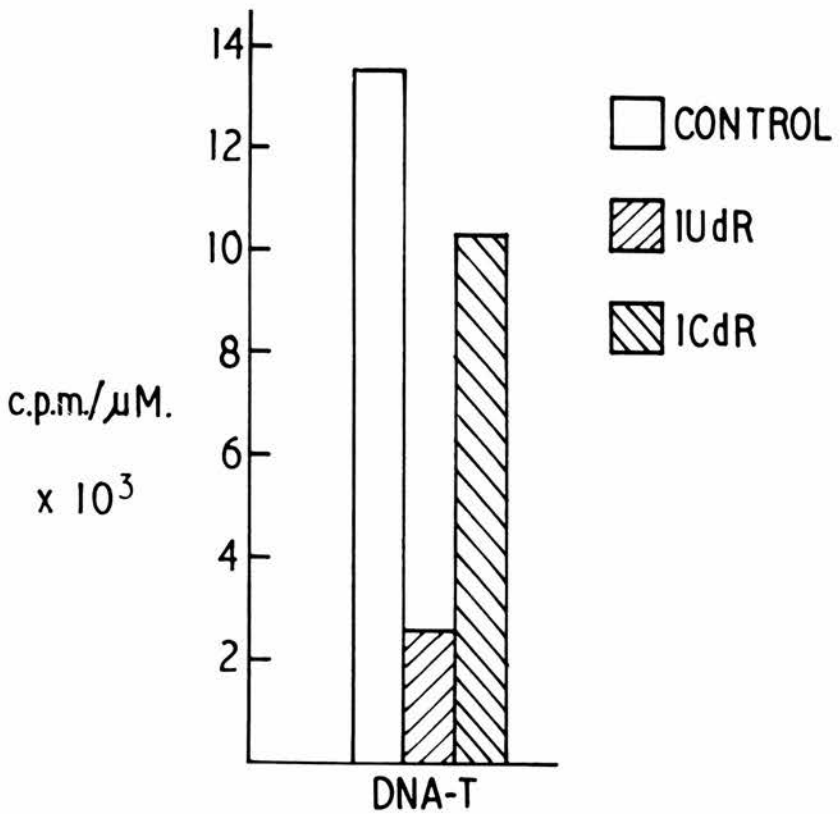


Table 18 and accompanying Histogram.

Effect of IUdR and ICdR on the utilisation of ^{14}C-formate for the biosynthesis of DNA-thymine in mouse lymphoma L5178Y cells *in vitro*

Analogue	DNA-thymine cpm/µm.°
None	13,500
IUdR	2,600
ICdR	10,300

° 1 cpm in amount of DNA corresponding to 1 mg. thymidine in the Stumpf reaction.



older and less active metabolically there was no evidence at all of inhibition of the synthesis of DNA-thymine by ICdR.

The relative inefficiency of ICdR compared with IUdR as an inhibitor of the synthesis of the phosphorylated derivatives of thymidine and DNA-thymine may indicate that the cells were lacking in the appropriate deaminase or that ICdR did not penetrate into the cell. The greater inhibitory effects observed with the compound in in vivo studies suggest that the compound may be deaminated elsewhere in the body e.g. in the liver or kidney, and subsequently acts as IUdR at the tumour site.

These initial studies suggest that weight for weight ICdR is less effective as an inhibitor of the synthesis of the thymine nucleotides and DNA-thymine than IUdR. The decreased toxicity, however, the increased solubility in water, and higher resistance to thermal decomposition in water of ICdR, in comparison with IUdR, offer significant advantages of this compound in the treatment of neoplastic disease.

SUMMARY.

Comparative studies have been made of the biological activities of IUdR and ICdR. The utilisation of ^{3}H-thymidine and ^{14}C-formate for the biosynthesis of DNA-thymine of L5178Y and Ehrlich ascites tumour cells in vitro is inhibited markedly by IUdR but to a much lesser degree by ICdR. Similarly the growth of Streptococcus faecalis

is inhibited markedly by IUdR but not at al by ICdR.

The growth of L5178Y lymphoma *in vivo*, however, is inhibited markedly by IUdR and to an almost equal degree by ICdR.

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SECTION III.

EFFECT OF DEOXYCYTIDINE ON THE RATE OF BIOSYNTHESIS OF PHOSPHORYLATED DERIVATIVES OF THYMIDINE AND DNA-THYMINE.

Introduction:

The utilisation of radioactive formate for the biosynthesis of the methyl group of DNA-thymine by mammalian cells *in vitro* has been shown to be increased by the nucleosides of uracil and cytosine, the latter having greater effectiveness (Prusoff, 1958a). It has been established in cell free systems that deoxyuridylic acid is an acceptor of the single carbon unit during the formation of TMP (Phear and Greenberg, 1957). Since both the riboside and deoxyriboside of cytosine were more stimulatory than the corresponding nucleosides of uracil, the possibility of the cytosine derivatives being an acceptor of the single carbon unit in addition to that of uracil was entertained (Prusoff, 1958a). The existence of 5-methyl cytosine derivatives in DNA (Wyatt, 1951a), as well as of a methyl cytosine deaminase (Wyatt, 1951b) was offered in support of this hypothesis. Biochemical evidence has since been reported of the greater stability of the cytosine nucleoside in comparison to the corresponding uracil derivative to metabolic degradation (Kriss and Revez, 1962) and hence the possibility of the greater effectiveness of the cytosine nucleosides merely by supplying a more stable depot of dUMP for condensation with the single carbon unit must also be considered in explanation of the results reported previously. An investigation has been made of the effect of deoxycytidine on the formation of TMP

TDP, TTP and DNA-thymine.

Time studies on the effect of Deoxycytidine on the utilisation of ^{14}C-formate for the biosynthesis of the phosphorylated derivatives of thymidine and DNA-thymine.

Murine lymphoblastic leukaemia L5178Y cells were harvested 6 days following the innoculation of AKR x DBA/2 F_1-hybrid mice.

The cells were prepared and incubated as described under methods (p.60-62). The results obtained are depicted in Figures 11, 12, 13 and 14. Figures 11 and 12 represent an experiment in which the cells were first examined after 5 mins. and Figures 13 and 14 represent a similar experiment in which samples were taken from 0.5 mins. onwards. The striking feature is that within 1 min. of incubation in the presence of deoxycytidine a marked increase was observed in the various thymine nucleotide fractions over those in the control cells. Also the TMP fraction within this period of time contained most of the radioactivity derived from formate. This is in agreement with the concept that formylation occurs at the nucleoside monophosphate level. These results do not present evidence in support of an acceptor role for deoxyuridylic acid to the exclusion of deoxycytidylic acid. Deoxycytidine presumably following phosphorylation could accept the single carbon unit and then become deaminated, whereas the accepted pathway for which evidence is available would suggest that following phosphorylation, deamination by deoxycytidylic deaminase occurs prior to the acceptance of the single carbon moiety. The data presented are in agreement with either interpretation and until evidence is presented in support of the existence of a formylated cytosine

Figure 11.

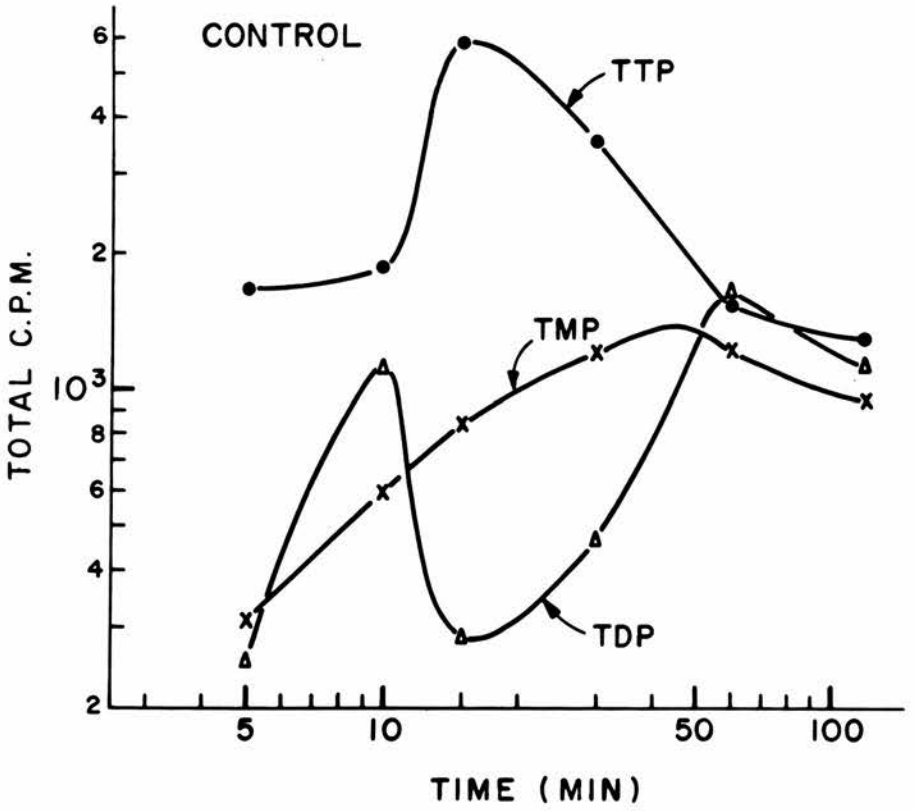


Diagram showing total radioactivity in each of the thymine nucleotide fractions following incubation of mouse lymphoma L5178Y cells with ^{14}C-formate for increasing periods of time beginning from 5 mins.

Figure 12.

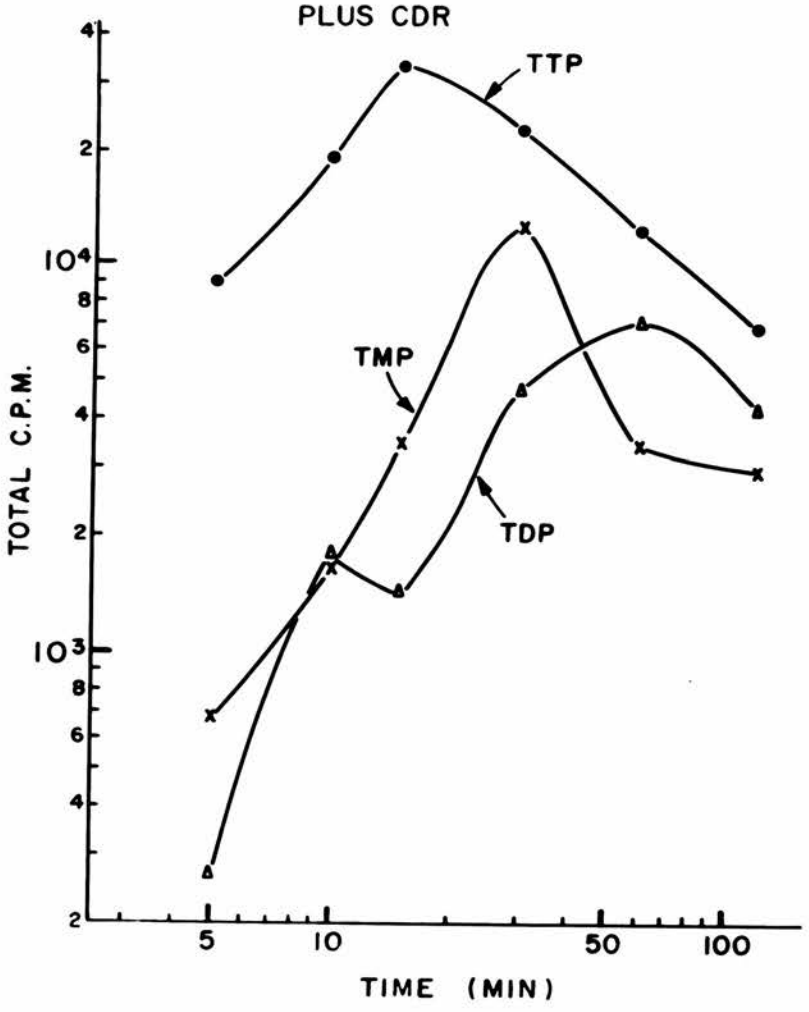


Diagram showing total radioactivity in each of the thymine nucleotide fractions following incubation of mouse lymphoma L5178Y cells with ^{14}C-formate and deoxycytidine for increasing periods of time beginning from 5 mins.

Figure 13.

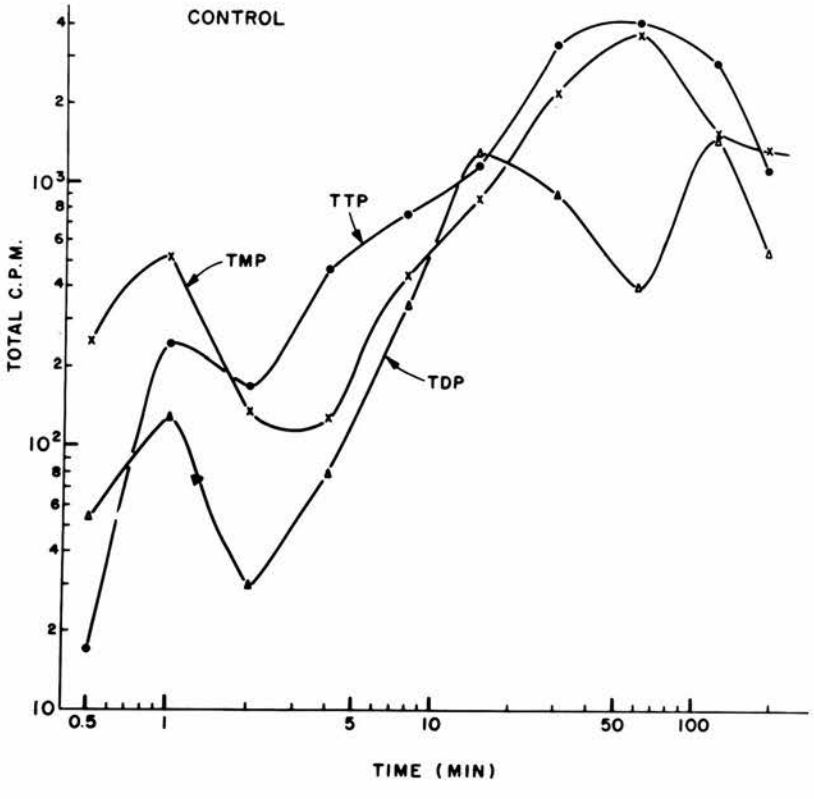


Diagram showing total radioactivity in each of the thymine nucleotide fractions following incubation of mouse lymphoma L5178Y cells with ^{14}C-formate for increasing periods of time beginning from 0.5 mins.

Figure 14.

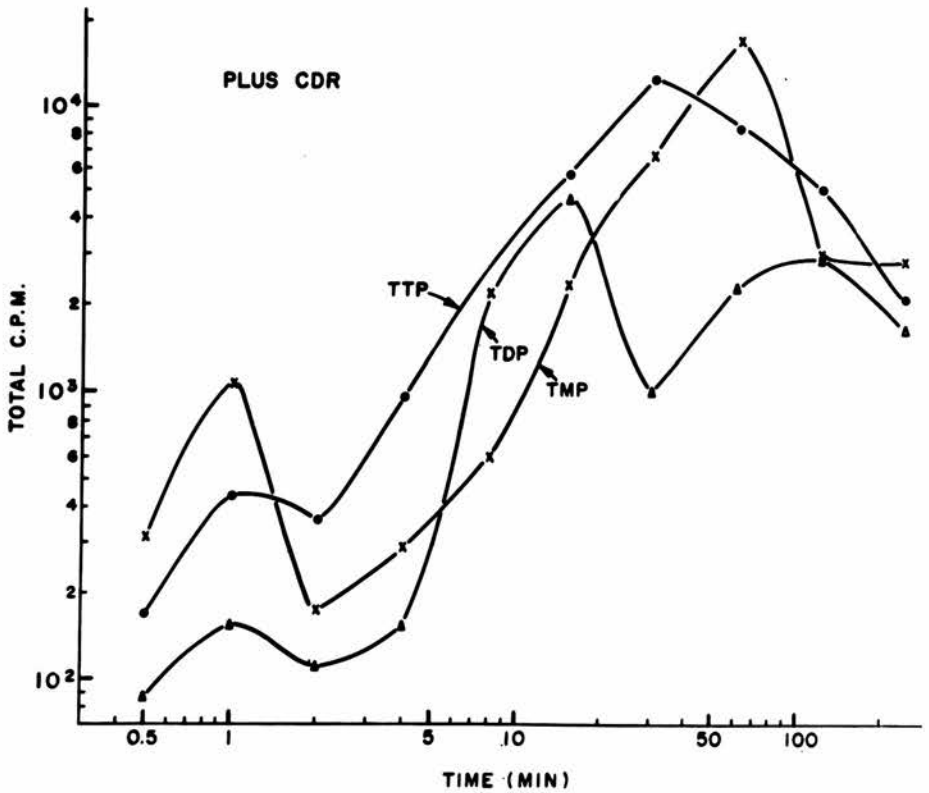


Diagram showing total radioactivity in each of the thymine nucleotide fractions following incubation of mouse lymphoma L5178Y cells with ^{14}C-formate and deoxycytidine for increasing periods of time beginning from 0.5 mins.

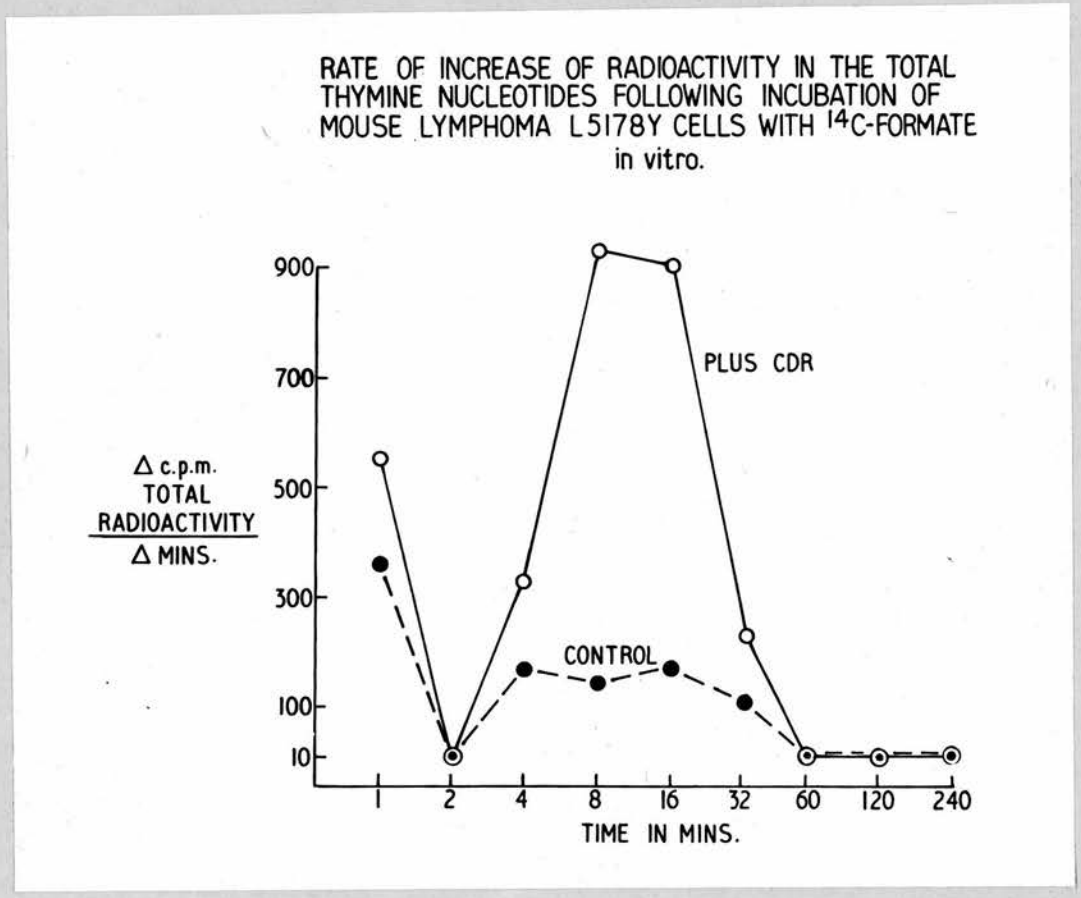
nucleotide intermediate, it must be assumed that deoxycytidine exerts its stimulatory effect via deoxyuridylic acid and not via a formylated cytosine intermediate.

Although the distribution of radioactivity in the TMP, TDP and TTP fractions derived from cells incubated in the presence and absence of deoxycytidine at the various intervals of the time studied were essentially similar, there was a marked increase in the absolute amount of radioactivity when incubated with deoxycytidine.

From Figures 13 and 14 may be seen that there are two dips in each of the curves. The first occurs at approximately the same point in time in each fraction (i.e. at about 1 minute) and there are several possible explanations. It might be related to the beginning of DNA synthesis which may possibly have been delayed due to previous cooling of the cells or some other unknown factor. If this were so, it would probably be necessary to postulate the formation of oligonucleotides which were present in the acid soluble fraction but possessed of too strong a charge to be eluted from the column along with the TTP fraction.

The second dip in the curve occurred in all instances after 30 minutes and here the explanation is equally difficult. From Figure 15 in which the total radioactivity in the three fractions (TMP, TDP and TTP) is plotted against time it is clearly demonstrated that the rate of formation or accumulation of nucleotides drops precipitously after 16 minutes and probably indicates that catabolic reactions are playing a large part in determining the distribution of radioactivity. Since DNA is still being synthesised at the same time,

Figure 15.


RATE OF INCREASE OF RADIOACTIVITY IN THE TOTAL THYMINE NUCLEOTIDES FOLLOWING INCUBATION OF MOUSE LYMPHOMA L5178Y CELLS WITH ^{14}C-FORMATE in vitro.
Δ c.p.m. TOTAL RADIOACTIVITY / Δ MINS.
900
700
500
300
100
10
PLUS CDR
CONTROL
1
2
4
8
16
32
60
120
240
TIME IN MINS.

(Table 19, Fig. 16), the interpretation becomes very difficult.

In Figure 17 the percentage of the total radioactivity in each fraction is plotted as a function of time during the first 16 minutes. It may be noted that the TMP and TTP curves are roughly reciprocal. As the percentage radioactivity falls in the TMP fraction it rises in the TTP fraction suggesting the possibility of the conversion of TMP to TTP without the formation of TDP. Moreover, the rise in radioactivity in the TDP fraction coincides with a decrease in the TTP fraction. Both of these phenomena are consistent with the suggestion of Bianchi that TMP is converted to TTP with the subsequent production of TDP by dephosphorylation of TTP. Whether TDP is actually on the metabolic pathway or not can only be determined by the measurement of the specific activity of the nucleotide fractions.

Effect of deoxycytidine on the capacity of IUdR to inhibit the utilisation of ^{14}C-formate for the biosynthesis of the phosphorylated derivative of thymidine and DNA-thymine.

It has already been shown that IUdR markedly inhibited the utilisation of ^{14}C-formate for the formation of TMP, TDP, TTP and DNA-thymine in the L5178Y cells *in vitro* (p. 81). In view of the increased formation of thymine nucleotides in the presence of deoxycytidine, it was of interest to determine whether deoxycytidine could reverse the inhibitory effects of IUdR. In these cells the prime metabolic site of inhibition had been shown previously and confirmed here to be in the conversion of TMP to higher phosphorylated nucleotides. The results are shown in Table 20 and its histogram.

Again IUdR resulted in a small but definite decrease in the

Table 19 and Figure 16.

The effect of deoxycytidine on the utilisation of radioactive formate for the biosynthesis of DNA-thymine by murine lymphoma L5178Y leukaemic cells in vitro

Time of Incubation. min.	DNA-thymine. (cpm/µm). Control.	Plus CdR.
4	24	180
8	200	570
15	1,300	3,000
30	2,000	5,800
60	4,600	10,200
120	10,300	20,200
240	20,300	30,400

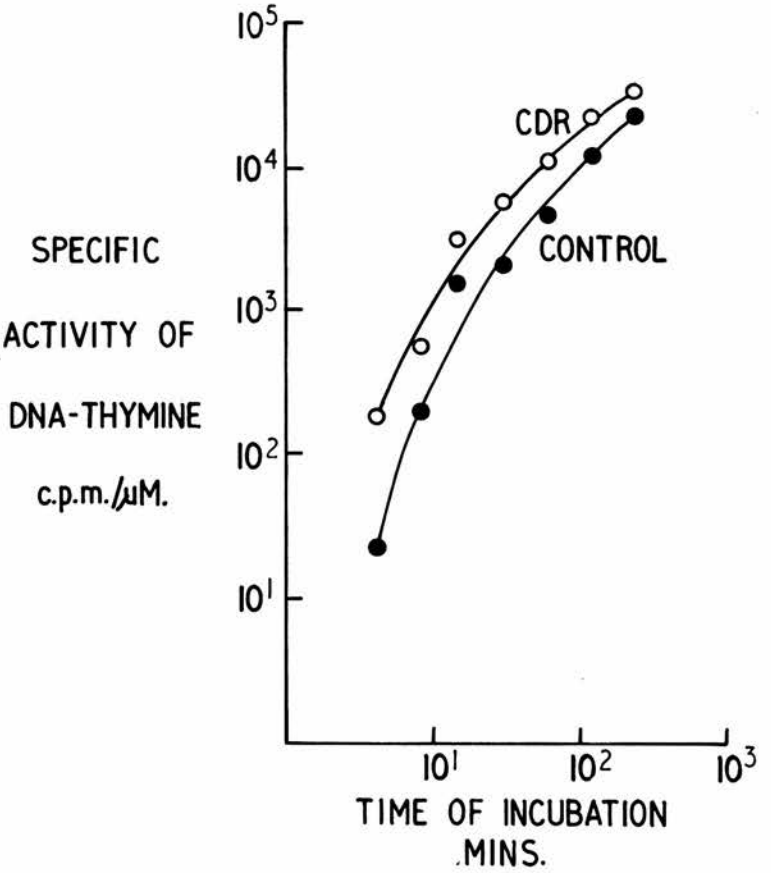


Figure 17.

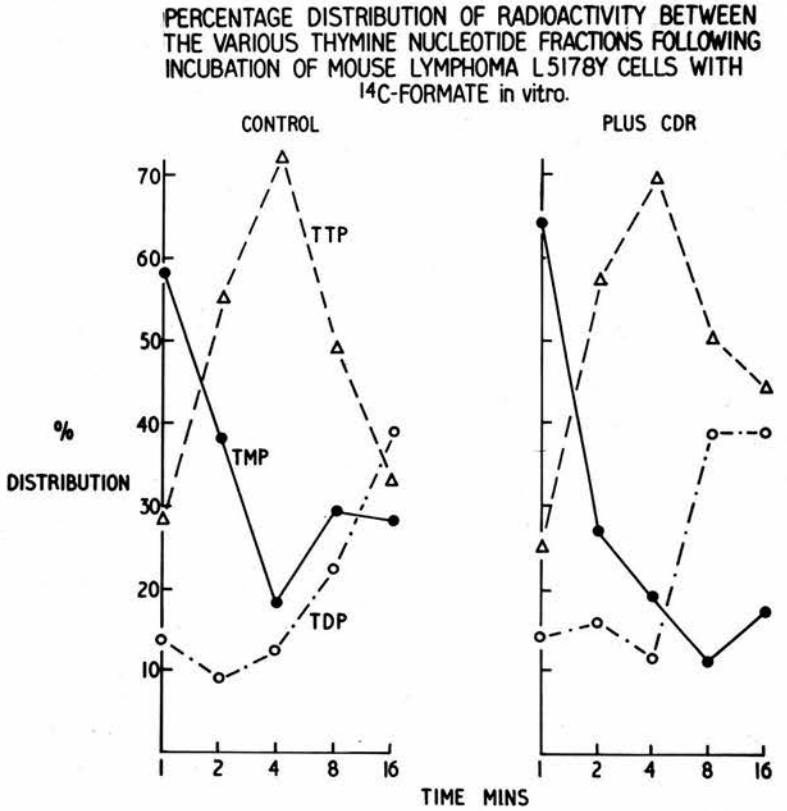
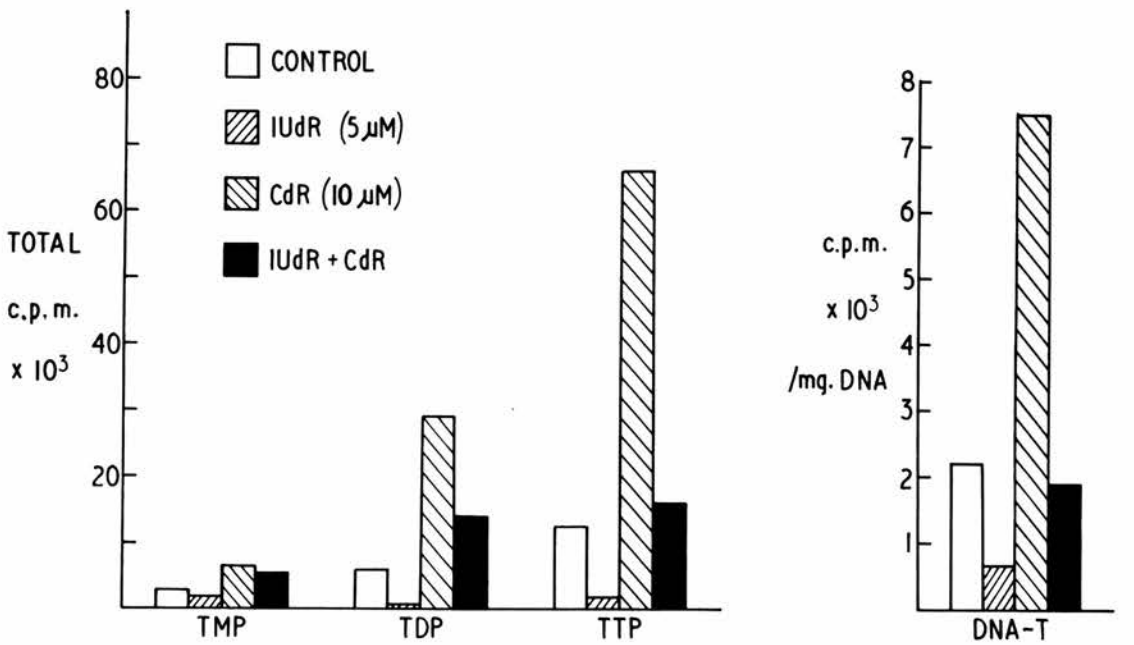

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY BETWEEN THE VARIOUS THYMINE NUCLEOTIDE FRACTIONS FOLLOWING INCUBATION OF MOUSE LYMPHOMA L5178Y CELLS WITH 14C-FORMATE in vitro.
CONTROL
PLUS CDR
%
DISTRIBUTION
70
60
50
40
30
20
10
TTP
TMP
TDP
1
2
4
8
16
TIME MINS

Table 20 and accompanying Histogram.

Effect of deoxycytidine on the inhibition by IUdR of the utilisation of ^{14}C-formate for the biosynthesis of TMP, TDP, TTP and DNA-thymine by mouse leukaemia 5178 in vitro

Addition	TMP cpm.	TDP cpm.	TTP cpm.	DNA-thymine. cpm./mg DNA.
None	2900	5900	12,400	2200
IUdR (5 µm.)	1900	680	1,800	650
CdR (10 µm.)	6500	29,000	66,000	7,500
IUdR + CdR	5300	14,000	16,000	1,900



amount of TMP formed in the presence or absence of deoxycytidine, but a more marked inhibition in the formation of the higher phosphorylated thymine nucleotides (TDP and TTP). The inclusion of IUdR, in the presence or absence of deoxycytidine, resulted in approximately 75 per cent inhibition in the specific activity of DNA-thymine. However, the absolute specific activity of DNA-thymine as well as the individual nucleotide pools derived from cells incubated in the presence of IUdR and deoxycytidine were markedly higher than those derived from the non-inhibited cells incubated in the absence of deoxycytidine. Thus, deoxycytidine did prevent in part the inhibitory effects of IUdR, presumably by increasing the size of the TMP pool.

SUMMARY.

A study has been made on the effect of deoxycytidine on the biosynthesis of the phosphorylated derivatives of thymidine and DNA-thymine in murine lymphoma L5178Y cells in vitro. In each of these fractions the amount of ^{14}C-formate incorporated was increased approximately two to three fold in the presence of deoxycytidine at all intervals of time up to a 4 hour incubation period. Although no conclusive results can be drawn an analysis of the amount and distribution of radioactivity in the various fractions at intervals of 0.5 mins. to 4 hrs. suggests that TMP is formed from dUMP and then converted directly to TTP without the intervention of TDP which may be formed later by dephosphorylation of TTP. Deoxycytidine has again been shown to be a precursor of DNA-thymine and TMP appears to be the primary product, but whether deamination occurs before or after

methylation is undetermined. The pattern of inhibition of the synthesis of the thymine nucleotides produced by IUdR was not altered by deoxycytidine but the absolute inhibitory effects were partially prevented, presumably by the increased formation of TMP.

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CHAPTER VII.

GENERAL DISCUSSION.

In order to assess the clinical value of IUdR and ICdR in the chemotherapy of neoplastic disease much more experience with the compounds is needed. Both drugs are extremely expensive, however, and the supply is consequently very restricted at the moment.

Three major clinical aspects may be considered in the evaluation of the potential effectiveness of an antimetabolite, the use of the drug alone, the use of the agent in combination with radiotherapy and combination therapy with other suitable antimetabolites. In the light of its biochemical activity, IUdR offers possibilities in all three directions. It is capable of blocking an important metabolic pathway and of actually altering the composition of the DNA. This not only causes the death of some cells but also gives rise to increased sensitivity to radiation injury. Finally, the synergistic inhibition of both the biosynthesis de novo of TMP and its incorporation into DNA through the use of a combination of FUdR and IUdR has aroused great interest.

Although IUdR is extensively incorporated into the DNA polymer, it has not been established that inhibition of cellular reproduction is causally related to this observed effect. The decrease in the biosynthesis in thymine nucleotide fractions consequent upon administration of IUdR may be of relevance. Many pyrimidine nucleotides are known to function as co-enzymes but no such role has so far been shown to exist for the thymine nucleotides in mammalian

cells. That such functions do exist, however, cannot be excluded particularly in view of the fact that they have been implicated in the synthesis of the bacterial cell wall (Okazaki, 1960; Kornfeldt and Glasor, 1960). ICdR following deamination, appears to act in a manner similar to IUdR but whether or not it has, in addition, other actions which are peculiar to the cytidine derivative is as yet undetermined.

The effects of IUdR appear to be exerted on rapidly growing tissues of all kinds and so far not enough difference has been noted between its inhibitory action in normal and neoplastic cells to produce the desired results. In view of the reports of higher levels of dCMP-deaminase activity in certain experimental tumours (Maley et al, 1959) as compared with normal tissues, however, ICdR may hold out more hope for a selective attack upon neoplastic tissue, since it may be preferentially phosphorylated and deaminated by these malignant cells. This hope was not borne out in the experiments reported above but this does not exclude the possibility of such an effect with certain tumours in man.

Although only very preliminary studies have been made on the extent of uptake of IUdR into DNA of human tumours, it appears that under the dosage regime so far employed only very small amounts have been incorporated (Welch, 1961). This is probably to be expected since not more than about one third of the cells are synthesising DNA at any one time and the exposure of these cells to IUdR has been only for a few hours. Higher levels of incorporation might be obtained if the drug were administered slowly over long periods of

time, particularly if given intra-arterially in the particular vessel supplying the neoplasm. Whether increased incorporation of the drug would, in fact, enhance its effectiveness as an antitumour agent, still has to be proved.

Of great interest is the capacity of at least some of the halogenated pyrimidines to sensitise cells to x-irradiation. The clinical usefulness of these compounds is that differential radiosensitisation of neoplasm might be achieved by coupling the selective affinity of such agents for dividing cells with localised irradiation which would thus confine the enhanced effect to the treatment field and spare normal rapidly dividing tissue such as marrow and intestine (Djordjevic and Szybalski, 1960). As Kaplan et al (1962) point out, however, if "bifilar" labelling of DNA is really essential for radiosensitivity to occur then the tumour would have to be allowed to double in size and then replicate its DNA once more in preparation for doubling again in the presence of the analogue before the radiosensitivity of any of its cells would be significantly enhanced. Fortunately, the necessity of "bifilar" labelling has been seriously challenged so that the possibility of combination therapy with X-rays serving as a useful clinical tool still remains, as some of the <u>in vivo</u> studies already carried out would suggest.

An approach to combination therapy which these authors suggest is the alternate use of the analogues and irradiation. These procedures, if spaced at optimum intervals, could label and then selectively destroy a small proportion of tumour cells at each cycle leaving behind the more radioresistant cells to be labelled in a

subsequent cycle. If such an approach enabled complete sterilisation of the tumour with as little as a 10 per cent reduction in total dose of X-rays it would represent a very real advance, since the margin of safety in many radiotherapeutic situations is small.

Whether or not an effective agent for the control of cancer will be found, it is certain that through continued studies of the metabolic reactions affected by the agents under discussion and others like them, important contribution will be made to our knowledge of the fundamental processes of the cell, both normal and neoplastic. Moreover, other uses will no doubt be found for certain of them other than that for which they were originally designed. It has recently been demonstrated (Kaufman, 1962; Perkins et al, 1962; Corrigan et al, 1962) that IUdR when administered locally is effective against the virus of herpes simplex as it affects the eye. Such infections are extremely difficult to treat and may lead to corneal scarring and blindness. If, therefore, these claims are substantiated by future workers, IUdR will be an exceedingly valuable drug and one of the few which has any action at all on virus infections.

The most rational approach to chemotherapy would appear to be based on an essential biochemical difference of a quantitative nature between neoplastic cells and their normal counterparts. One of the best examples of such an approach is the marked success achieved with the sulphonamide compounds which inhibit the biosynthesis of folic acid by certain microorganisms by competing with para-aminobenzoic acid for essential enzymes. The mammalian host meanwhile is unaffected because, unlike the microorganisms, it is capable of utilising preformed folic acid ingested in the food for participation in various metabolic reactions. Unfortunately, analogous quantitative differences have

not so far been found in neoplastic cells as compared with normal cells. However, it is doubtful whether new chemical reactions would be expected to appear in the probable conversion of a normal cell to a neoplastic one. It would appear to be more probable that certain biochemical features are deleted and that such cells no longer respond to their environmental controlling influences in the same way as the normal cell. Quantitative differences, however, are seen in some tumours as compared with normal tissues. One such example is the ability of certain tumours to carry on aerobic as well as anaerobic glycolysis at sustained high levels with the production of large amounts of lactic acid from glucose.

In future cancer research the biochemistry of the malignant cell in humans is worthy of much more investigation. If characteristic metabolic pathways can be elucidated it may be possible to design compounds with a specific action. In this event each type of tumour could be treated with the drug best able to interfere with the particular biochemical reactions concerned.

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SUMMARY OF THESIS.

The biosynthesis of the phosphorylated derivatives of thymidine and of DNA-thymine has been reviewed. The compounds which interfere with specific reactions on the metabolic pathways concerned have been discussed and particular attention drawn to the halogenated pyrimidines which behave as analogues of uracil or thymine or their derivatives. Such compounds inhibit the synthesis of DNA-thymine and in consequence DNA-synthesis and cellular reproduction is often impaired. The halogenated pyrimidines may themselves be incorporated into DNA in lieu of thymine with the formation of fraudulent DNA and in the cells concerned a reduction in the threshold of radiation sensitivity may ensue. The available information on the clinical effects of the halogenated pyrimidines both on tumour growth and on radiation sensitivity has been summarised.

Studies on the thymidine analogues 5-iodo-2'-deoxyuridine (IUdR) and 5-iodo-2'-deoxycytidine (ICdR) have been described. Investigations were undertaken to determine the effect of these analogues on the formation and utilisation of the phosphorylated derivatives of thymidine and on the formation of DNA-thymine in various murine and human neoplastic tissues. Both compounds reduced the incorporation of ^{3}H-thymidine or ^{14}C-formate into DNA-thymine as a result of the inhibition of the utilisation of thymidine, thymidylic acid or thymidine triphosphate and the specific

metabolic site primarily affected in the various tissue studied was a characteristic of the individual tissue.

Comparative studies with IUdR and ICdR were undertaken both in vitro and in vivo. The mechanism of action of ICdR (following deamination) was found to be similar to that of IUdR but it appeared to be less effective as an inhibitor of the synthesis of DNA-thymine and it had little or no effect on the growth of Streptococcus faecalis, an organism whose growth is severely inhibited by IUdR. In mice, however, the compound was only slightly less effective than IUdR in the inhibition of tumour growth, in vivo.

The effect of deoxycytidine on the biosynthesis of the phosphorylated derivatives of thymidine and DNA-thymine has also been studied in murine lymphoma L5178Y cells in vitro. In each of these fractions the amount of ^{14}C-formate incorporated was increased two to three fold in the presence of deoxycytidine. Although no conclusive results could be drawn on analysis of the amount and distribution of radioactivity in the various fractions after periods of incubation which varied from 0.5 mins. to 4 hours, it appeared that deoxycytidine increased the rate of formation of thymidylic acid which was then converted directly to thymidine triphosphate without the intervention of thymidine diphosphate which may be formed later by diphosphorylation of the triphosphate.

Deoxycytidine was shown to reduce the inhibitory effects of IUdR on the biosynthesis of the thymine nucleotides and DNA-thymine.

A brief discussion is given on the antimetabolites under study with suggestions for their possible clinical application in the future.

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COMMUNICATIONS.

In connection with the experimental work reported in this thesis it was considered desirable to make the following communications to learned societies and journals.

COMMUNICATIONS TO LEARNED SOCIETIES.

(1) Studies on the mechanism of action of 5-iodo-2'-deoxyuridine.
I.W. Delamore and W.H. Prusoff. American Assoc. for Cancer Research, April, 1961.

(2) The effect of deoxycytidine on the rate of biosynthesis of the phosphorylated derivatives of thymidine and DNA-thymine.
I.W. Delamore and W.H. Prusoff. Scottish Society of Experimental Medicine, October, 1961.

(3) Studies with the thymidine analogue, iododeoxyuridine, on murine and human leukaemic cells.
I.W. Delamore and W.H. Prusoff. Medical Research Society January, 1962.

PUBLICATIONS.

(1) Separation of thymidine and its mono-, di- and tri- phosphate by paper chromatography.
I.W. Delamore and W.H. Prusoff. Biochemical Pharmacology 8, 336, (1961).

(2) Effect of 5-iodo-2'-deoxyuridine on the biosynthesis of phosphorylated derivatives of thymidine.
I.W. Delamore and W.H. Prusoff. Biochemical Pharmacology 11, 101, (1962).